Mycorrhizal colonization and growth characteristics of salt stressed *Solanum lycopersicum* L.

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Abstract

The present study aimed to examine the effects of root colonization in tomato, *Solanum lycopersicum* L. cv. Moneymaker, by the arbuscular mycorrhizal (AM) fungus, *Glomus intraradices* Shenck and Smith, on alleviating salt stress. I postulated that AM symbiosis increases tomato plant performance to salt stress. Two greenhouse experiments were done according to a randomized factorial experimental design. The results showed a significantly higher level of AM root colonization that also occurred earlier in salt than non-salt treated plants. There were also positive interactions between root colonization levels and the alleviation of salt stress; these contributions resulted initially on higher root fresh mass (FM), later on shoot FM, and DM, and higher phosphorus and unchanged potassium concentrations in roots. The effects observed in salt-treated plants were significant when root colonization levels were significantly different than those observed in non-salt treated plants. This suggests a relationship between the level of root colonization and the alleviation of salt stress in plants. The attempt to use molecular techniques to detect early root colonization was quite successful in detecting the presence of *G. intraradices* in AM plants. However, it was not possible to detect the presence of the AM fungus as early as by classical root staining. This was observed presumably because sampling methods were different. In general, the results support the hypothesis that AM root colonization contributes to some extent to salt resistance of tomato plants.

Résumé

La présente étude visait à déterminer les effets de la colonisation des racines de la tomate, *Solanum lycopersicum* L. cv Moneymaker, par le champignon mycorhizien à arbuscules (MA), *Glomus intraradices* Shenck et Smith, sur la réduction du stress salin. J'ai postulé que la symbiose MA augmente la performance des plantes de tomate au stress salin. Deux expériences en serre ont été réalisées selon un design expérimental factoriel randomisé. Les résultats ont montré un niveau de colonisation des racines par le champignon MA significativement plus élevé et qui est apparu plus tôt chez les plantes stressées au sel par comparaison aux plantes témoins. Des interactions positives ont aussi été observées entre le niveau de colonisation des racines et la réduction du stress salin, contribuant ainsi à une masse fraîche (MF) supérieure des racines, puis celle des parties aériennes ; en général les masses fraîches et sèches ont augmenté avec la colonisation MA. De plus, la concentration de phosphore était plus élevée et celle du potassium constante dans les racines MA. Les effets observés chez les plantes en conditions salines, ont été significatifs lorsque les niveaux de colonisation des racines étaient significativement différents de ceux observés chez les plantes témoins. Ceci suggère une relation entre le niveau de colonisation des racines et la réduction du stress salin. L'utilisation de techniques moléculaires pour détecter les étapes initiales de la colonisation racinaire a été moins efficace que la méthode de coloration des racines, possiblement en raison des méthodes d'échantillonnage différentes. En général, les résultats soutiennent l'hypothèse que la colonisation MA contribue à la résistance au sel des plantes de tomate.

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I would like to dedicate this thesis to Minako Sato

“The greatest oak was once a little nut who held its ground.”

Anonymous

Table of Contents

Chapter 3: Results

List of Figures

List of Tables

List of Appendices

List of Abbreviations

AMF	Arbuscular mycorrhizal Fungus
AM-	Non-inoculated
AM+	Inoculated
ANOVA	Analysis of variance
DM	Dry mass
DNA	Deoxyribonucleic acid
FM	Fresh mass
ICP-ES	Inductively coupled plasma emission spectrometry
NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
PLVG	Polyvinyl-alcohol-lactic-glycerol

Chapter 1

Introduction

Environmental factors can act as stressors that impact the evolution of living organisms on Earth (Schluter 2001). Indeed, survival necessitates the ability to rapidly adapt to changes in the environment, especially those which represent long term or chronic changes. Whenever possible, one of the easiest ways to counteract such stresses is to relocate to a more suitable niche (Huey et al. 2002). However, such a strategy is obviously restricted in a short term period and is not achievable with stationary organisms such as plants. Consequently, plants have developed a variety of strategies to cope against biotic stresses such as herbivory or parasitism, and abiotic stresses such as salinity, drought, heat or toxic metal contamination (Hodges et al. 1995; Subramanian and Charest 1998, 1999; Audet and Charest 2006, 2008, 2009).

Among abiotic stresses, soil salinization is probably one of the most important in the world (Hasegawa et al. 2000; Zhu 2003). It was observed many centuries ago that agricultural soils became infertile over time because of increased irrigation (Schilfgaarde 1994). Historical findings indicated that several societies relying on irrigated fields failed due to salinization, Mesopotamia is one example (Jacobsen and Adams 1958; Araus et al. 2007). Some records also show that a switch between crops was made to alleviate this problem, for example from wheat, a salt sensitive species, to barley, a salt tolerant one (Araus et al. 2007). However, such strategy was of limited benefit since with time barley yields decreased. These effects led to relocation and deadline of populations as

happened in Mesopotamia (Weiss et al. 1993). Even nowadays, with the advent of modern agricultural practices and new management measures, the loss of crops due to field salinization remains a major concern. The main reason why agricultural fields are affected by salt is generally due to continued irrigation and a lack of sufficient drainage resulting in waterlogged soils which leads to increased surface salt concentrations as a consequence of evaporation (Ritchie et al. 1972). Consequently, a reduction in crop yields, mainly because of osmotic stress, as well as nutritional and toxic effects, occurred. It is estimated that at least one third of all irrigated agricultural lands are affected to some degree by salinity (Williams 1999). Furthermore, the increasing demands in food production constantly push agricultural fields to areas where water and soils have naturally or not high salt levels (Araus et al. 2007).

Salt stress disrupts the process of osmosis causing water to diffuse out of the plant leading to wilting and eventually death. Under such conditions, roots are the first line of defense when exposed to salinity and act as filtering organs (Narendra 2007). This osmotic change also disrupts mineral transport and thus the acquisition of sufficient minerals to ensure proper growth. Salinity was shown to reduce the uptake of nutrients, such as Ca and P, and to compete with others such as K. Most crop plants are glycophytes, *i.e.* sensitive to salt, and as a result they have a limited degree of tolerance to salt (Al-Karaki 2000). One adaptation towards salt tolerance is its sequestration into vacuoles even to toxic levels that could otherwise disrupt ionic balance (Zhu 2003; Parida and Das 2005). All these effects of soil salinity have led to research aimed at finding solutions to relieve crop stress and increase yield.

Plant species

Tomato, *Solanum lycopersicum* L., is widely distributed in a variety of climates, and is grown all around the Mediterranean Sea. Native to meso-America, tomato was first introduced in Europe by Spanish explorers after the discovery of the New World (Knapp and Jarvis 1990). Tomatoes are members of the *Solanaceae* also known as the nightshade family and were initially thought to be poisonous like their European members. The Spanish and Italian nations were the first Europeans to adopt this fruit in their culinary dishes and soon after tomatoes were widely distributed in Europe and all around the world. In 2008, the production of tomatoes reached over 130 million tons worldwide (Food and Agriculture Organization Statistic). Stress on tomato caused by excess salinity is of concern in the warm and dry mediterranean regions, where soil salinity occurs frequently due to inadequate irrigation management (Puigdefábregas and Mendizabal 1998; Rengasamy 2006).

Salinity and the biosphere

High soil salinity is a growing setback in agricultural development in many parts of the world, especially in arid and semiarid areas. Currently, high soil salinity occupies 7% of Earth's land surface and it is predicted that 50% of arable land will be affected by salinity by the half of the 21th century (Elevin et al. 2009). This could mostly occur due to soluble minerals found in irrigation water and the high fertilizer input from agricultural practices (Schilfgaarde 1994; Al-Karaki 2006). In addition, high temperature and low precipitation leading to salt accumulation at the soil surface affect the establishment, growth and development of plants and even more as salinity increases. The delay in root

growth can be caused by too low soil water potential and salt cell toxicity (Psarras et al. 2008). The latter causes cell death and root necrosis in the very sensitive genotypes. In addition to these deleterious effects on roots, growth of shoots is also affected and as a result the root/shoot ratio is disturbed (Maggio et al. 2007). Overall, salinity leads to many deleterious effects on plants and that at different life stages. To counteract this problem, many strategies were proposed to overcome salt detrimental effects such as searching for new salt-tolerant crops, genetically engineering plants, removing excessive salt accumulation in groundwater and desalinizing water for irrigation (Ashraf and Harris 2004; Flowers 2004; Zhang and Blumwald 2001). Although these strategies appear efficient, they are costly and out of reach for developing countries that are the most affected.

In nature, plants interact with several microorganisms such as bacteria and fungi that improve their performance when facing various environmental pressures. Indeed, most of terrestrial plants are involved in mutualistic associations with other organisms beneficial to both parties (Brundrett 2002). One of these associations is referred to as mycorrhiza, *gr.* fungus and root (Smith and Read 1997). There are numerous studies reporting that mycorrhizal associations lead to crop improvement, *e.g.* growth rate, biomass, and mineral uptake under saline or drought conditions (Augé 2004; Evelin et al. 2009; Subramanian and Charest 1998, 1999). Mycorrhizae were shown to have beneficial effects in delaying or coping with toxic effects caused by soil salinity by maintaining an overall physiological balance (Sharifi et al. 2007; Shokri and Maadi 2009). AM fungi occur naturally in saline environments despite the fact that they have a low

affinity with halophyte plants (Khan 1974). However, halophytes can benefit to some extent from AM symbiosis as in the case of *Phragmites australis,* for which the water content increased in salt AM plants (Al-Garni 2006). Interestingly, the most commonly observed AM fungus was among *Glomus* spp. (Landwehr et al. 2002). However, when comparing several *Glomus* spp., Porras-Soriano et al. (2009) observed that each AM fungal species has a different efficiency in alleviating plant salt stress.

Soil salinity can affect AM fungi by slowing down root colonization, spore germination, and hyphal growth (Juniper and Abbott 1993). Before colonization occurs, spores need to be hydrated in order to germinate which is difficult in saline soil. To some extent, salinity hampers AM fungi at early stages of the symbiosis which is delayed rather than inhibited (Juniper and Abbott 2006). However, other studies showed that there is in fact no reduction in AM colonization in the presence of NaCl (Aliasgharzadeh et al. 2001; Yamato et al. 2008), and even increases in sporulation and colonization occur (Peng et al. 2010). The discrepancies amongst studies suggest that various AM fungal spp. have varying tolerance to salinity, then questioning the host plant and AM fungus compatibility and tolerance (Porras-Soriano et al. 2009). These studies also suggest that AM fungal species have different capacities in protecting plants and that host compatibility might be an issue worth looking into when developing AM strategies in plant growth and tolerance under salt stress conditions.

Mycorrhizal fungi

Terrestrial plants have evolved many strategies to maximize their ability to absorb nutrients, especially under poor soil conditions. Mycorrhizal fungi date back

more than 450 million years ago (Bonfante and Genre 2008). Since then, they have been key elements for the growth of land plants. To maximize mineral absorption from nutrient poor soils, many plant species have developed symbiotic associations such as with AM fungi that are obligate symbionts, *i.e.* unable of completing their whole life cycle without host plants (Parniske 2008). In return, plants provide the AM fungi part of their carbohydrates (Pearson and Jakobsen 1993). The fungus can penetrate the root and initiate its differentiation into arbuscules, which are the main interface in nutrient exchange (Gianinazzi-Pearson 1996; Smith and Read 1997). Following further development of extraradical hyphae, the AMF surfaces from roots and actively forages soil nutrients (Read and Perez-Moreno 2003). The very fine hyphae spread over large soil volume and increase the absorption of essential nutrients that are often difficult for plants in poor soils (Smith and Read 1997).

Mycorrhizal life cycle

The establishment of mycorrhizal association involves several steps starting from recognition of the host root, followed by formation of the appressorium, penetration of epidermal cells, proliferation of intraradical hyphal, vesicular and arbuscular development, and extraradical hyphae and finally spore formation. The first step in recognizing the root involves the contact of hypha with the rhizoderm or root hair (Gianinazzi-Pearson et al. 2007). Then, the hypha develops an appressorium that is the binding point with its host (Marschner and Dell 1994). The appressoria then penetrate epidermal cells and make their way to cortical cells. Inside the root a network of hyphae is formed and gives rise to arbuscules; these highly branched structures are thought to

be the main interface for nutrient exchange between both partners (Muok and Ishii 2006). It was observed that both sucrose and phosphate transporters (Rausch et al. 2001) are present in the arbuscular membrane. Arbuscules have a finite life span that usually lasts a few days (Smith and Read 1997). Vesicles, found in the intercellular space, are structures that may withstand stresses such as freezing and dryness. They are able to survive in roots until soil conditions are favorable for re-growth. Hyphae can develop as an extraradical mycelium which is the main bridge between adjacent roots of the same or different plant species. This highly branched hyphal network scavenges minerals such as phosphorus and nitrogen and translocates them into the roots. It increases surface area uptake well beyond the root depletion zone. This makes the extraradical mycelium an important component in nutrient and water allocation to the host. It has been shown that hyphal network change the soil pH that facilitates the availability of elements such as P, Zn and Cu (Bago et al. 1996).

Mineral nutrition

Phosphorus

Salinity was shown to decrease P uptake as it precipitates with Ca, Mg and Zn, then being unavailable to plants (Evelin et al. 2009; Park et al. 2009; Wang et al. 2008). Consequently, P solubilization or added in fertilizer is required for plant growth. It was estimated that AM symbiosis helps the plant acquire up to 80% of its P requirement (Matamoros et al. 1999). Higher P uptake under saline conditions increases the plants ability of reducing any Na and Cl negative effects (Feng et al. 2002). Nutrient balanced

plants were shown to sequester these elements in vacuoles to maintain metabolic pathways and growth (Cantrell and Linderman 2001).

K/Na

In saline soils, plants tend to absorb more Na than K that compete for the same cell binding site (Rus et al. 2001). Even though this site cannot discriminate between ions, only one has a cellular function, as K is involved in the activity of a wide range of enzymes, operates stomatal movement and protein synthesis (Blaha et al. 2000). Salinity disrupts K**/**Na balance thus hampering plant growth. Grattan and Grieve (1998) showed that AM plants have higher K**/**Na ratio due to an increase of K uptake in shoot. Since AM colonization may increase plant growth, it then also reduces salt stress by growth dilution effect (Juniper et al. 1993). However, AM colonization was also shown to increase the Na uptake in *Distichlis spicata* (Allen and Cunningham 1983). With time, AM plants may accumulate Na through water uptake, then decrease it at high salt level. This implies that AM fungi may act as buffers from toxic conditions (Audet and Charest 2006).

Calcium

Calcium is essential as a second messenger among other functions. Under salt stress conditions, its concentration increases presumably to transduce signals (Cantrell and Linderman 2001). High Ca levels help plants to cope with salt stress as raising selectivity in K uptake and leading to better salt adaptation. Hence, Ca accumulation has been found to increase colonization and sporulation (Jarstfer et al. 1998).

Magnesium

Chlorophyll synthesis impaired by salt stress may reduce photosynthetic rate that however can be improved with Mg by AMF uptake (Giri and Mukerji 2004). A higher chlorophyll concentration has been shown in AM plants of lettuce under salt stress (Zuccarini 2007).

Water osmotic homeostasis

The water status in AM plants of *Jatropha curcas* L. was maintained at relatively normal levels under saline conditions (Kumar et al. 2010). Mycorrhizal colonization was shown to improve water conductance in roots and increase stomatal conductance thereby enhancing transpiration (Colla et al. 2008; Jahromi et al. 2008). AM colonization was also shown to lower osmotic potential by increasing plant compatible solutes. Several studies showed that AM symbiosis results in increasing nutrient uptake, photosynthetic rate and water status (Porras et al. 2009; Sheng et al. 2008; Zuccarini 2007).

My research goal was looking at the effects of colonization by *Glomus intraradices* Schenk and Smith on tomato, *Solanum lycopersicum* L., subjected to salt stress. The levels of salt, to which plants are exposed impact their lifespan as well as mycorrhization levels. It is expected that as salt concentration increases, plant growth and plant survival decrease. In contrast, if the level of stress imposed on the plant does not represent a high cost, an increase in mycorrhization levels will be observed, then indicating a positive investment towards the symbiotic relationship. I included in this study molecular biology to detect the presence of *G. intraradices* in roots. The nested

PCR should be more sensitive than root staining in detecting the AM fungus. Since root staining is usually done on plants that have been mycorrhized after a relatively long period it will not be able to detect any structure at the early stages of plant growth. The staining technique can determine colonization percentage of older plants (8 weeks and more) while the nested PCR can detect it earlier (before 8 weeks). Each of these two approaches can answer different questions towards a better understanding on how tomato plants and *G. intraradices* interact under salt stress.

Objectives, rationale and hypotheses

In this research, I examined the effects of colonization by *G. intraradices* on tomato subjected to salt stress conditions. My first objective was to determine proper conditions that allow both AM root colonization and plant growth under salt stress. Second, it was to determine the effects of salinity on a number of plant physiological parameters. Third, I investigated the time course needed for the establishment of AM colonization by using molecular techniques.

The rationale underlying this study was that tomato plants subjected to salt stress invest more in AM colonization to alleviate any deleterious effects. What is still unknown to date is how salinity impacts the early events of root colonization. My hypotheses were:

1. The AM root colonization level is higher in salt than non-salt treated plants and allows better plant growth.
2. The time course required for root colonization is shorter in salt than non-salt treated plants.

Chapter 2

Materials and Methods

Plant and AM fungus

The tomato, *Solanum lycopersicum* L. cv. Moneymaker (West Coast Seeds Ltd., BC, Canada) was used in this study. The AM inoculum consisted of *Glomus intraradices* Schenck and Smith (DAOM 181602) included in a fine granular substrate containing 15 propagules g^{-1} dry soil, and the non-AM control substrate contained filtrates of the rhizosphere microflora while excluding fungal spores (MYKE® PRO, Premier Tech, Rivière-du-Loup, QC, Canada).

Greenhouse conditions

Greenhouse experiments were conducted at the Center for Advanced Research in Environmental Genomics, University of Ottawa. Supplemental light was applied in the greenhouse at 736 mmol m^{-2} sec^{-1} for a 16-hour photoperiod and an average day and night temperature of 25°C. The relative humidity was set at 70% using an electronically managed water mist.

Experimental design

The randomized factorial design (1 plant x 2 AM x 2 NaCl x 2 times x 5 reps) used consisted of inoculated (AM+) or non-inoculated (AM-) plants subjected to salt (S+) or non-salt treatment (S-) for a total of 40 plants harvested after eight and ten weeks from germination.

Soil composition and growth procedure

The soil mixture consisted of sand:peat moss (1:1) homogenized with an industrial mixer, autoclaved for 20 min at 121°C (Century SG-120 sterilizer), and allowed to cool before potting. Both substrates were obtained from Ritchie's Feed & Seed Inc., Ottawa.

Before sowing, 3 L of soil mixture were soaked with water and used to fill the bottom of each pot. Depending on the treatment, each pot was then added with 1 L of inoculated (AM+) or non-inoculated (AM-) substrate. One litre of soil mixture was used to complete the volume to 5 L. Four seeds were sown and let grown until germination (7-9 days). Plantlets were allowed to grow for one week until the appearance of the first true leaf after which only one plant per pot was allowed to grow. Plants were watered as needed and fertilization was applied bi-weekly until harvest with 100 mL of a modified Long Ashton nutrient solution (Hewitt and Smith 1975; Appendix 1). During the entire time of the experiment, no leaching was allowed to avoid any loss of water, nutrient or salt.

Salt treatment

To determine the proper salt level, two trials were previously done using different NaCl (ACS reagent, ≥99.0%) concentrations (50, 100 and 150 mM). In the first trial, the salt concentrations were added after sowing; in the second one, after the first true leaf emerged. These concentrations were too high and most plants died after a few

weeks in both trials. This led us to readjust the NaCl concentration to 17mM which was used in the two greenhouse experiments as described below.

In the first experiment, the salt treatment was initiated after the appearance of the first true leaf (one week after germination). This allowed me to observe how an early salt stress can affect tomato plants. The salt treatment was done by giving two doses of 17 mM NaCL (5 g NaCl in 200 ml dH_2O) whereas the non-salt control plants received 200 mL of water. After the 5^{th} week, salt treatment was terminated and all the plants received the same amount of water and fertilizer until the end of the experiment. In the second experiment, the main difference from the first one, was that the salt treatment was administered at a single time (17 mM NaCl) following germination. I compared here root colonization levels in salt and non-salt treated plants in order to determine whether salinity has an impact on the ability of AM colonization to be established. In both greenhouse experiments, the root colonization level, shoot and root fresh mass (FM) and dry mass (DM) were measured. In addition, the root/shoot ratio and the AM effect on shoot and root performance from both FM and DM (see *plant performance*) were determined.

The total density of AM fungal structures, *e.g.*, hyphae, arbuscules and vesicles, was also determined. These structures are present at different stages during the symbiosis establishment providing us with information about density changes under saline conditions. The nested PCR (polymerase chain reaction) was used to detect early

root colonization as described below. Chlorophyll *a* and *b* analysis (Knudson et al. 1977) was done to detect any effect of salt and mycorrhizal treatments.

Harvest

From each plant, the shoot was cut from roots and put in a paper bag after taking its fresh mass. The root material was then removed from the soil, washed vigorously with tap water to remove soil particles, then dried with paper towel. The total root fresh mass was measured, and a part of root material was stained to determine AM colonization level. All fresh mass material was taken on the same day of the harvest. To determine dry mass, fresh material was put in an oven for three days at 70°C. The dry plant material was later used for mineral analysis and nested PCR analysis.

Chlorophyll analysis

In the second experiment, the chlorophyll analysis was determined from one gram of fresh leaf tissue placed in a 50 ml eppendorf with 99% ethanol. This was left in the dark overnight until it lost completely its green color. An aliquot of 2 ml was placed on a spectrophotometer plate and read at 663nm and 649nm (Knudson et al. 1977). The leaf material was removed from the ethanol solution and dried. Chlorophyll concentrations were expressed as µg chl/mg DM as following:

$$\frac{\mu g\ Chl\ a}{ml\ solution} = (13.70)(Abs\ 665nm) - (5.76)(Abs\ 649nm)$$

$$\frac{\mu g\ Chl\ b}{ml\ solution} = (25.80)(Abs\ 649nm) - (7.60)(Abs\ 665nm)$$

Root staining

Roots from four plants per treatment were stained according to Vierheilig et al. (2005). One gram of fresh root tissue per plant was cleared in 2.5% KOH solution at 90°C for 20 min under fume hood. Cleared roots were then rinsed 3 times with tap water and put in a 5% blue ink (Sheaffer) solution diluted in 5% acetic acid at 95°C for 3 min. Roots were allowed to destain for 20 min in tap water after which a few drops of acetic acid were added to terminate the destaining. Stained roots were stored at room temperature until determination of AM colonization percentage.

Percentage of colonization

To assess the percentage of colonization, 100 x 1 cm root fragments were mounted on slides (10 per slide) in polyvinyl-alcohol-lactic-glycerol (PVLG) medium. Each fragment was examined under a compound microscope (10X and 40X) for the presence of arbuscules, vesicles, hyphae or spores (if any). Mycorrhizal colonization percentage was reported as the total number of fungal structures observed in all root segments.

Density of AM fungal structures and plant performance

The density corresponds to the total number of AM fungal structures observed per root cm. Similarly to root colonization %, a segment of stained root was mounted on a compound microscope then a count of the AM structures was done. From the count of each of these structures, their density was determined as number of structures per cm and then converted as a percentage based on total density:

$$total\ density\ = \frac{number\ of\ all\ AM\ structure}{root\ cm}$$

$$AM\ structure\ density\ = \frac{number\ of\ specific\ AM\ structure}{root\ cm}$$

$$density\ (\%) = \frac{AM\ structure\ density}{total\ density} \times 100$$

To assess the AM colonization contribution on plant growth, the following equation (Gange and Ayres 1999) was applied:

$$AM\ plant\ performance\ = \frac{M - NC}{NC}\ \times 100$$

M: mean performance of mycorrhizal plants

NC: mean performance of non-mycorrhizal plants.

Mineral analysis

The mineral analysis (Ca, Cu, Fe, K, Mg, Mn, P, Zn and Na) was done using ICP-ES (Inductively Coupled Plasma Emission Spectrometry) in the laboratory of Dr. N. De Silva, Dept. of Earth Sciences, University of Ottawa. One gram of dried tissue (root or shoot) per plant (n= 4) was ground into a fine powder and 1 mg of each was put in 1 ml of concentrate 12 M nitric acid (HNO_3) at 90°C for 2 hours. This material was then diluted in 10ml of milliQ water (Millipore continental water system) before analysis. To assess how salt conditions might change the concentrations of minerals such as potassium known to reduce deleterious effect of sodium, I have calculated K/Na ratios.

Mycorrhizal DNA extraction

Each DNA extraction was performed on a single root segment based on manufacturer's recommendation (E.Z.N.A.® Plant DNA Kit, cat. #D3485-02). DNA extraction was performed from dry material (max of 10mg). From each plant, 3 root segments were taken for DNA extraction. The negative and positive controls were defined as non-mycorrhizal and mycorrhizal roots, respectively. DNA extracts were subsequently either stored in a freezer at -20°C or used immediately for nested PCR amplification.

PCR primers

PCR amplification was used to determine whether AM root colonization could be detected earlier than by the staining method. To verify the presence of *G. intraradices* after DNA extraction, PCR primers were designed using available data from the National Center for Biotechnology Information (NCBI). Sequences specific to mitochondrial *G. intraradices* DNA absent from root sequences were selected. This led to the design of the following specific primers: (A1) (5'-ATCAGGTCTCTCACGGCTAAAG-3') and (A2) (5'-CTGGTAAACCAGAGGTCACTCC-3') directed against part of the large ribosomal subunit (accession: FJ648425). The expected size of the amplicons was 822bp. This sequence was then used as a template for a second amplification in order to perform the nested PCR. The second set of primers (B1) (5'-AGGATAACCTTGTGGAAAGCAC-3') and (B2) (5'-ACTCCATTTAAGTCCTCGCGTA-3') were expected to generate an amplicon of 423bp.

PCR reaction

Each PCR reaction was prepared in a 25 µL final volume which consisted of water, Taq PCR buffer (1x), forward primer (0.2µM) and reverse primer (0.2 µM), $MgCl_2$ (3mM), dNTP mix (200 µM) and finally Taq polymerase (0.05u/µL). The DNA templates were either 1 µL of extracted DNA or in the case of the nested PCR, 1 µL from the first PCR reaction (after a 1:10 dilution). PCR was carried out as follows: (denaturation 3 min at 95°C, annealing 1 min at 58°C, elongation 30 sec at 72°C) X 1, followed by (30 sec at 93°C, 30 sec at 58°C, 30 sec at 72°C) X 35 and a final elongation step of 5 min at 72°C followed by a final hold at 4°C. The same program was used for the nested PCR using the second set of primers. After each PCR, electrophoresis on 1% agarose gels was performed on the same day to verify the presence of *G.iIntraradices* DNA.

Statistical analysis

Statistical analyses were done for each parameter after verifying normality and evenness of variance by performing Kolmogorov-Smirnov and Levene's tests, respectively. Analysis of variance (ANOVA) was coupled with Bonferroni or Tukey multiple mean comparison tests. All the data were analysed using S-PLUS® 8.0 (Insightful Corp 2007).

Chapter 3

Results

3.1 First experiment

Mycorrhizal colonization

The one-way ANOVA indicated that the salt treatment has a significant ($P<0.001$) effect on root colonization percentage that was significantly three times higher in salt than non-salt treated plants after eight weeks of growth, and tended to be two times higher after ten weeks (Fig. 1).

Shoot and root fresh and dry mass and root/shoot ratio

For shoot fresh mass (FM), there were significant effects of AM ($P<0.001$) and salt ($P=0.009$) treatments, and their interaction ($P=0.031$). In salt treatment, the shoot FM was two times higher in AM than non-AM plants after eight weeks, but not after ten weeks, and remained similar among the non-salt treated plants (Fig.2). There were also significant effects ($P<0.001$) of both AM and salt treatments on shoot dry mass (DM) that was twice higher in AM than non-AM salt treated plants after eight weeks (Fig.3). For root FM, three-way ANOVA indicated significant effects of AM ($P=0.03$), salt ($P<0.001$) and time ($P<0.001$), and interactions of AM with time ($P=0.037$) and salt ($P=0.003$). In fact, mycorrhization significantly increased root FM in salt treated plants after eight weeks (Fig. 4). After ten weeks, regardless of the AM treatment, the root FM was significantly higher in non-salt than salt treated plants, as for the root DM after

eight weeks. There was a strong effect of salt treatment (P<0.001) on root DM. Over both harvests, the DM of non-salt treated roots doubled and was the same in salt treated roots (Fig. 5). There were significant effects of AM (P=0.025) and salt (P<0.001) treatments on root/shoot ratio that was lower in AM salt than non-AM non-salt treated plants after eight weeks (Fig. 6).

Shoot and root AM colonization performance

The AM colonization performance was defined as the effect on shoot and root mass, as follows = [(AM – Non-AM/Non-AM) x 100]. The ANOVA indicated a significant (P=0.004) effect of salt on shoot FM performance which was significantly three times higher in salt than non-salt treatment (Fig. 7A). There were also significant effects of salt (P<0.001) and time (P=0.028) on root FM performance (Fig. 7B), which was significantly higher in salt than non-salt treatment at eight weeks. On shoot DM, salt treatment had a significant effect on AM performance (P<0.001). After eight weeks, it was significantly three times higher in salt than non-salt treatment (Fig. 8A), not after ten weeks. The salt treatment (P<0.001) and its interaction with time (P=0.02) were significant on root DM performance that was significantly three times higher in salt than non-salt treated plants after eight weeks (Fig. 8B).

Figure 1: AM colonization percentage (%) of tomato roots under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Bonferroni's test.

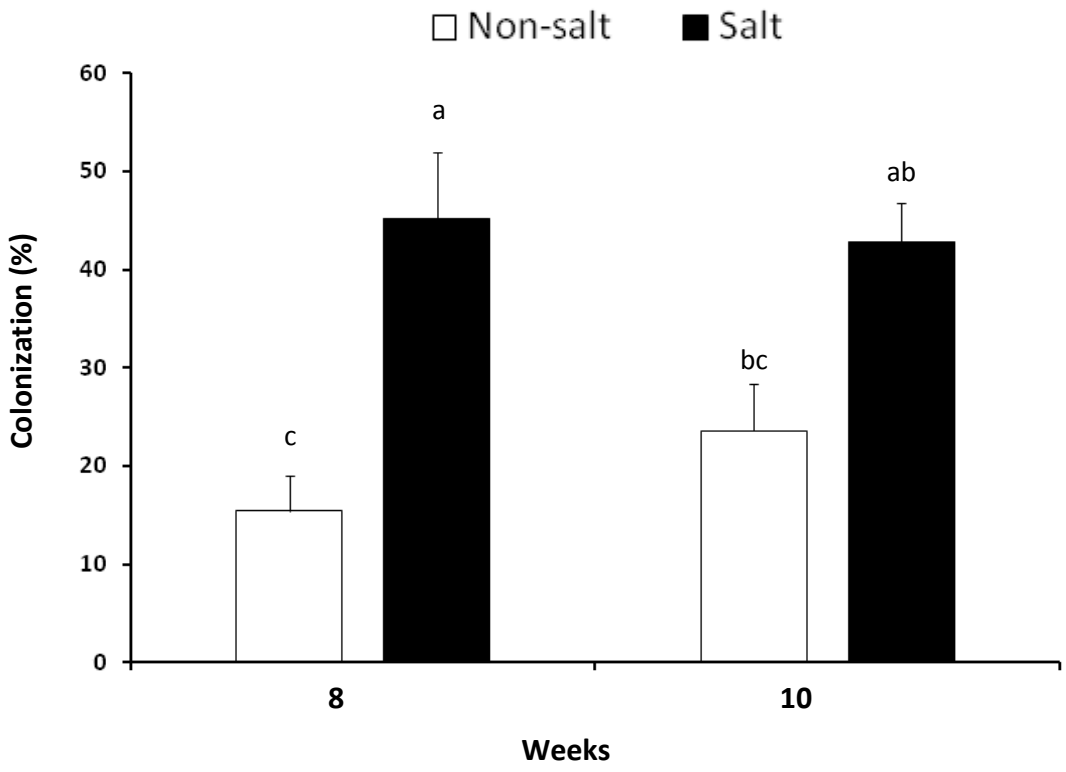

Non-salt
Salt
60
50
40
30
20
10
0
Colonization (%)
a
ab
bc
c
8
10
Weeks

Figure 2: Shoot fresh mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

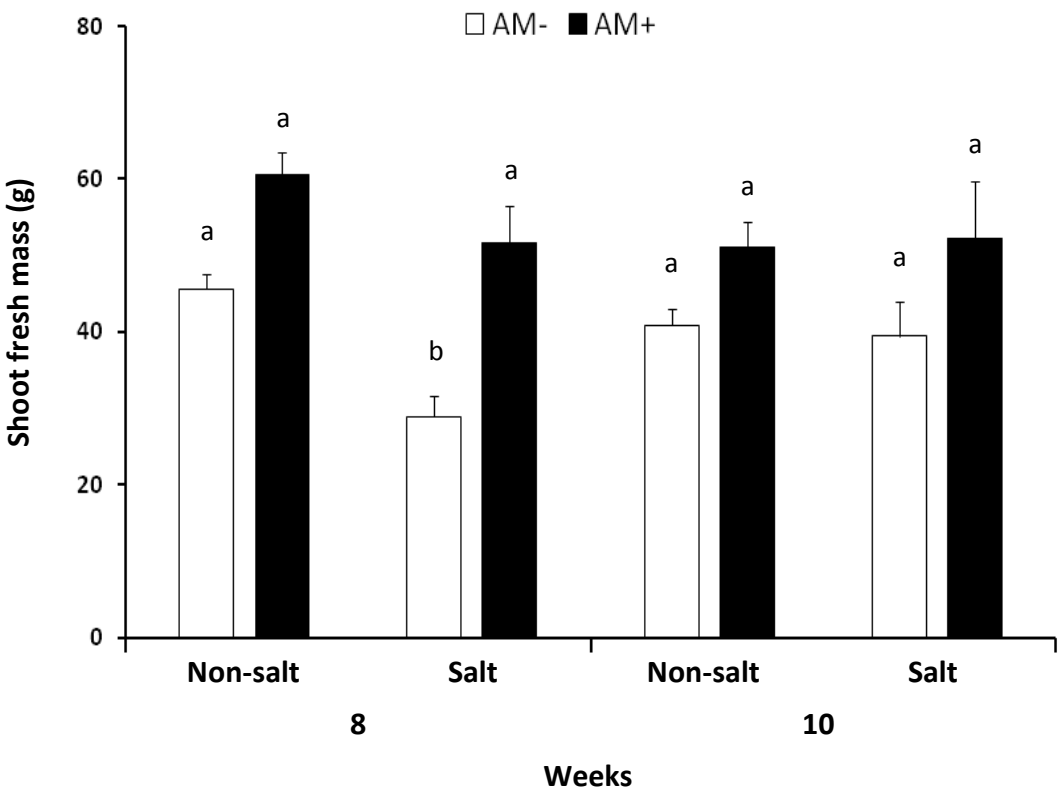

AM-
AM+
Shoot fresh mass (g)
80
60
40
20
0
a
a
b
a
a
a
a
a
Non-salt
Salt
Non-salt
Salt
8
10
Weeks

Figure 3: Shoot dry mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Tukey's test.

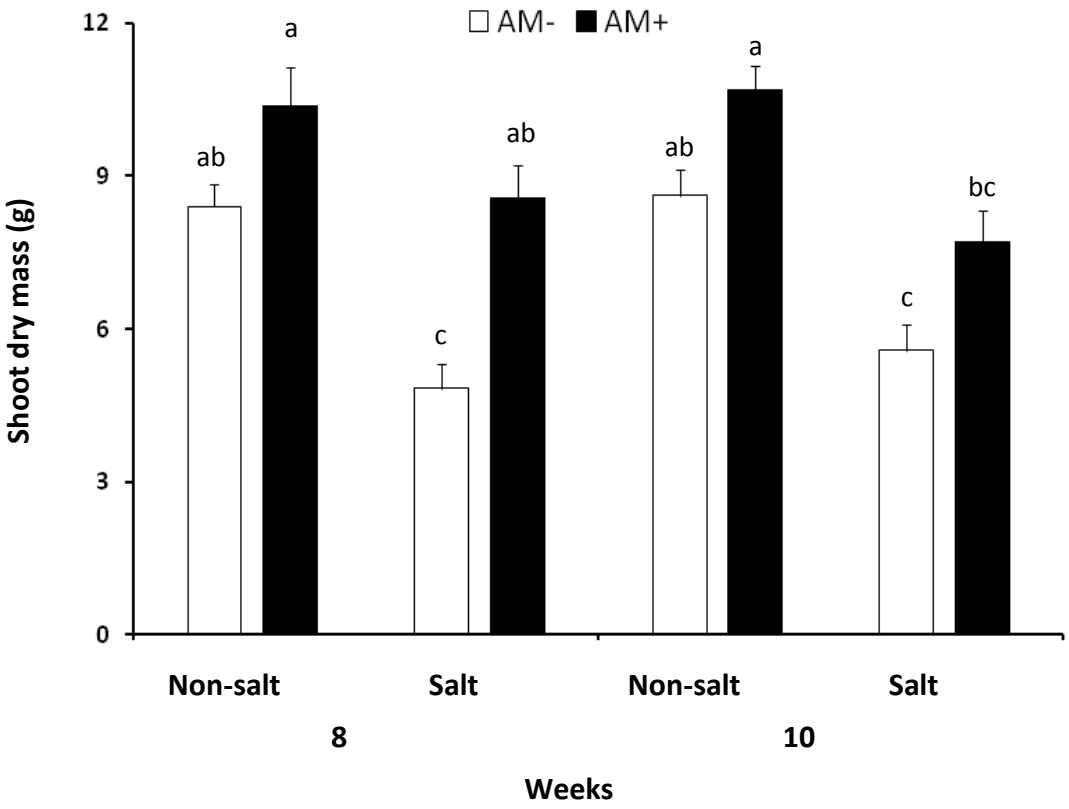

AM-
AM+
Shoot dry mass (g)
12
9
6
3
0
a
ab
c
ab
ab
a
c
bc
Non-salt
Salt
Non-salt
Salt
8
10
Weeks

Figure 4: Root fresh mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Bonferroni's test.

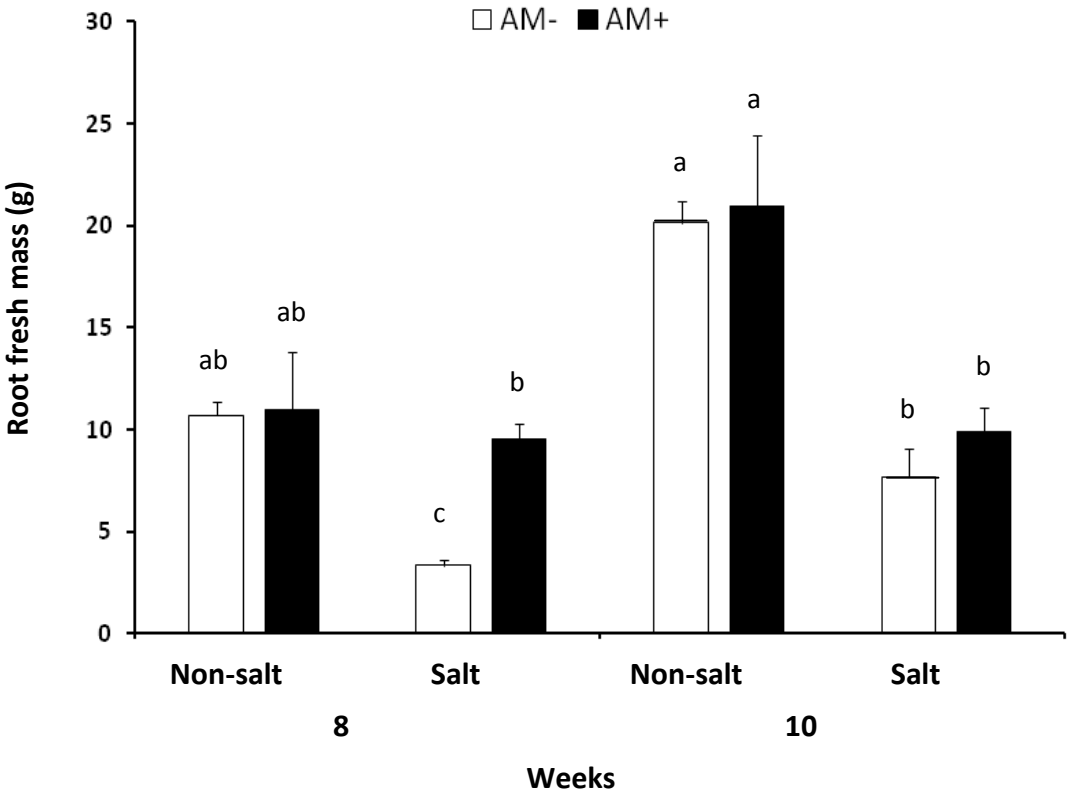

□ AM- ■ AM+
Root fresh mass (g)
30
25
20
15
10
5
0
ab
ab
c
b
a
a
b
b
Non-salt
Salt
Non-salt
Salt
8
10
Weeks

Figure 5: Root dry mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

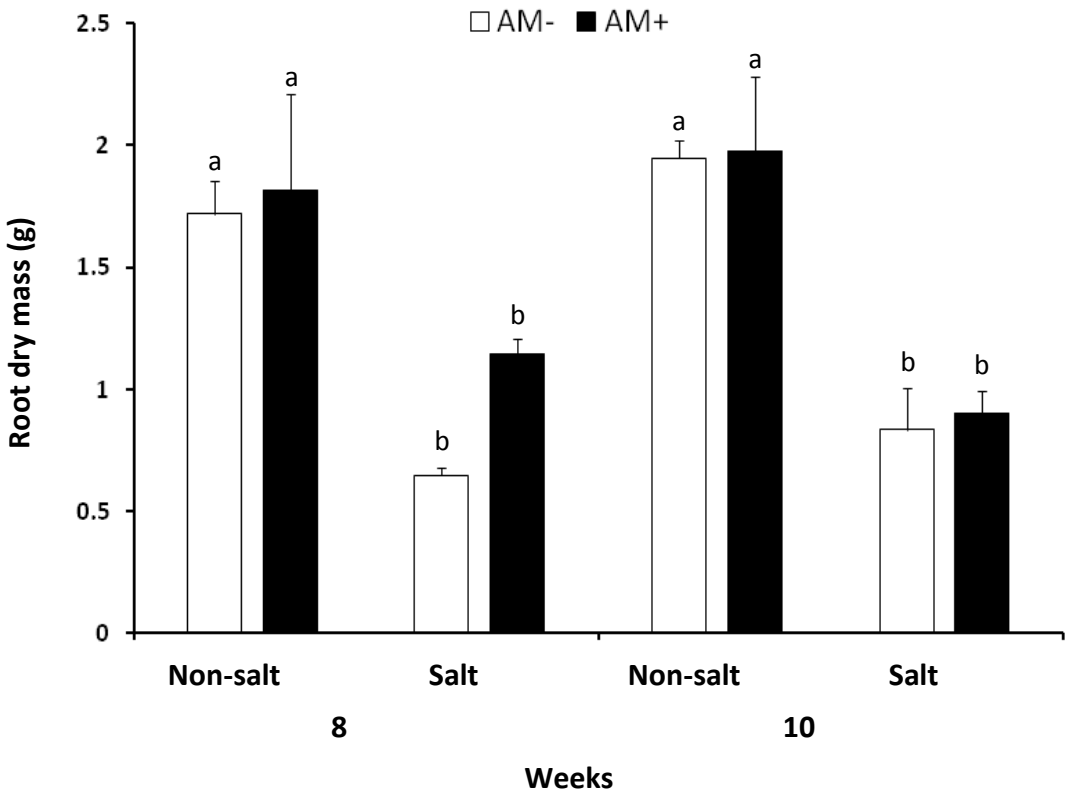

AM-
AM+
Root dry mass (g)
2.5
2
1.5
1
0.5
0
a
a
b
b
a
a
b
b
Non-salt
Salt
Non-salt
Salt
8
10
Weeks

Figure 6: Root/shoot ratio of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Tukey's test.

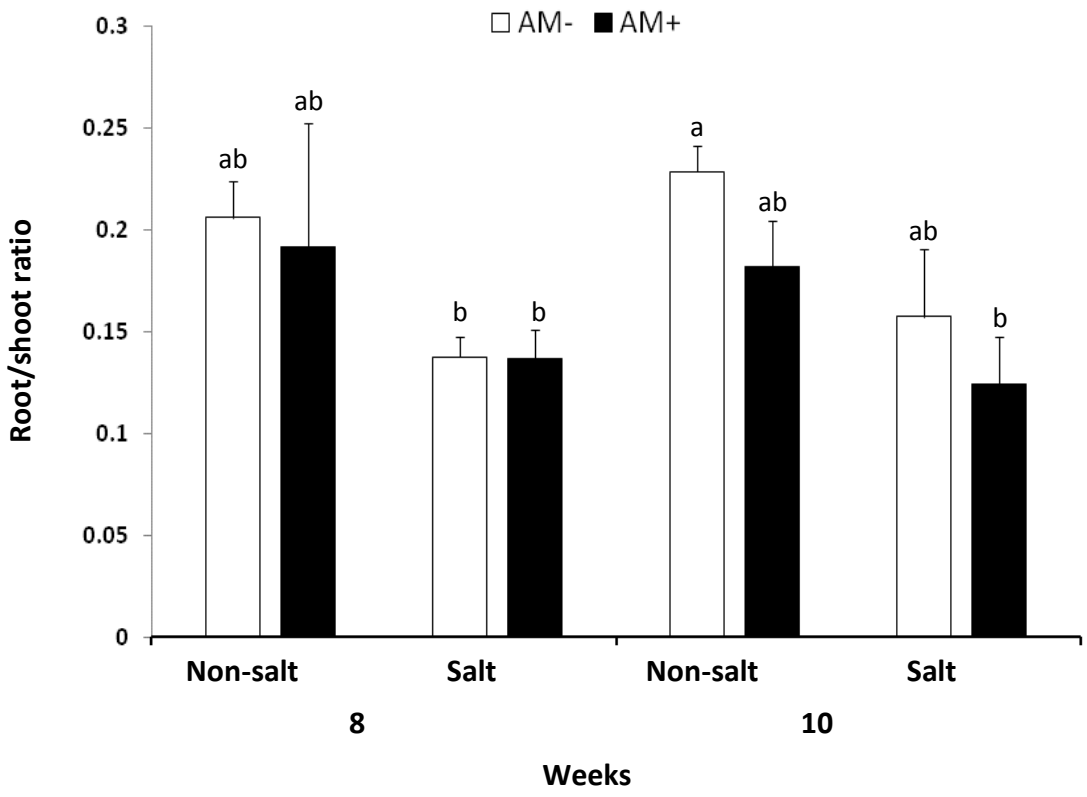

□ AM- ■ AM+
0.3
0.25
0.2
0.15
0.1
0.05
0
Root/shoot ratio
ab
ab
b
b
a
ab
ab
b
Non-salt
Salt
Non-salt
Salt
8
10
Weeks

Figure 7: Effect (%) of AM over non-AM colonization on the fresh mass (FM) of shoots (A) and roots (B) of tomato plants under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Tukey's test.

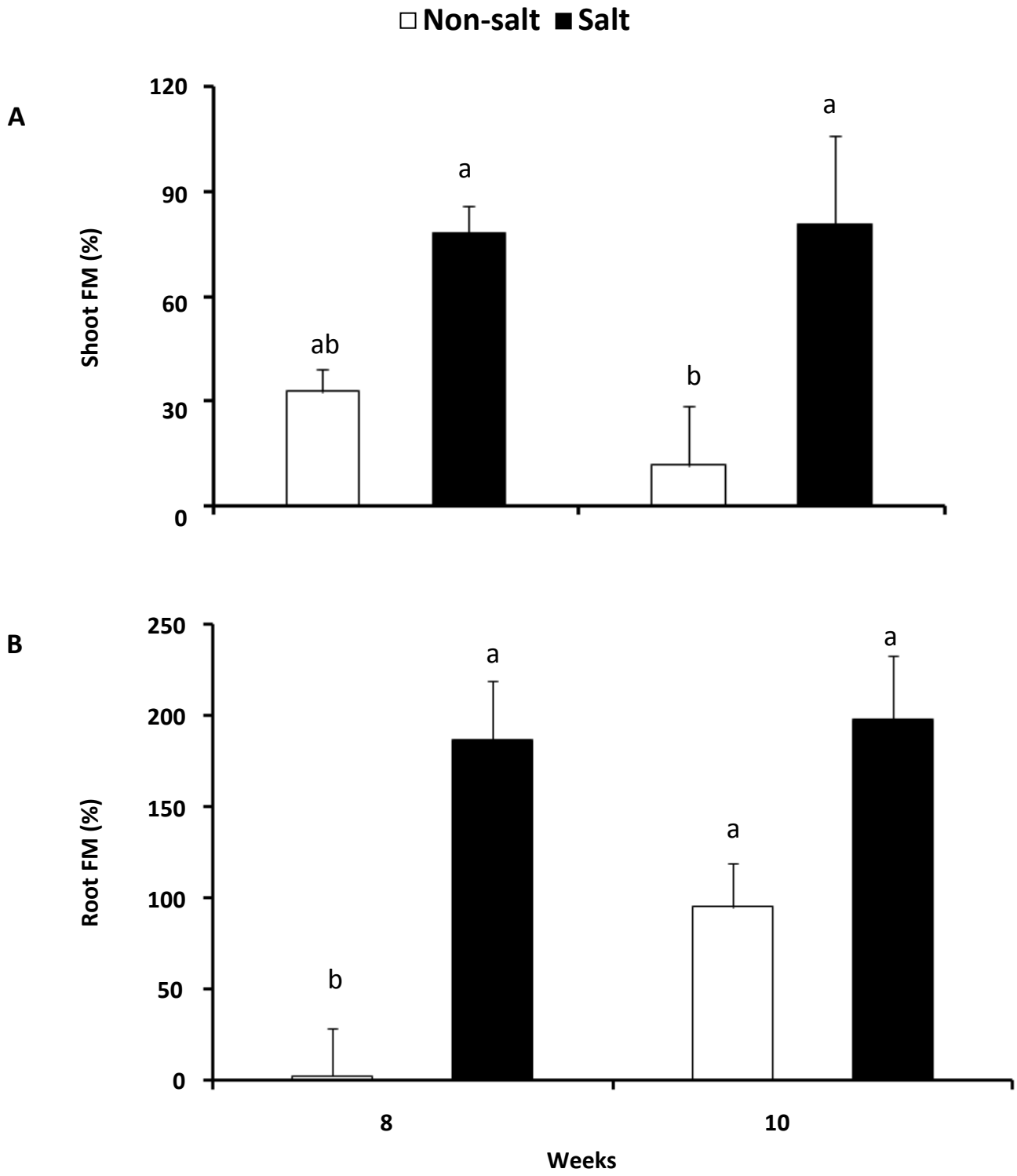
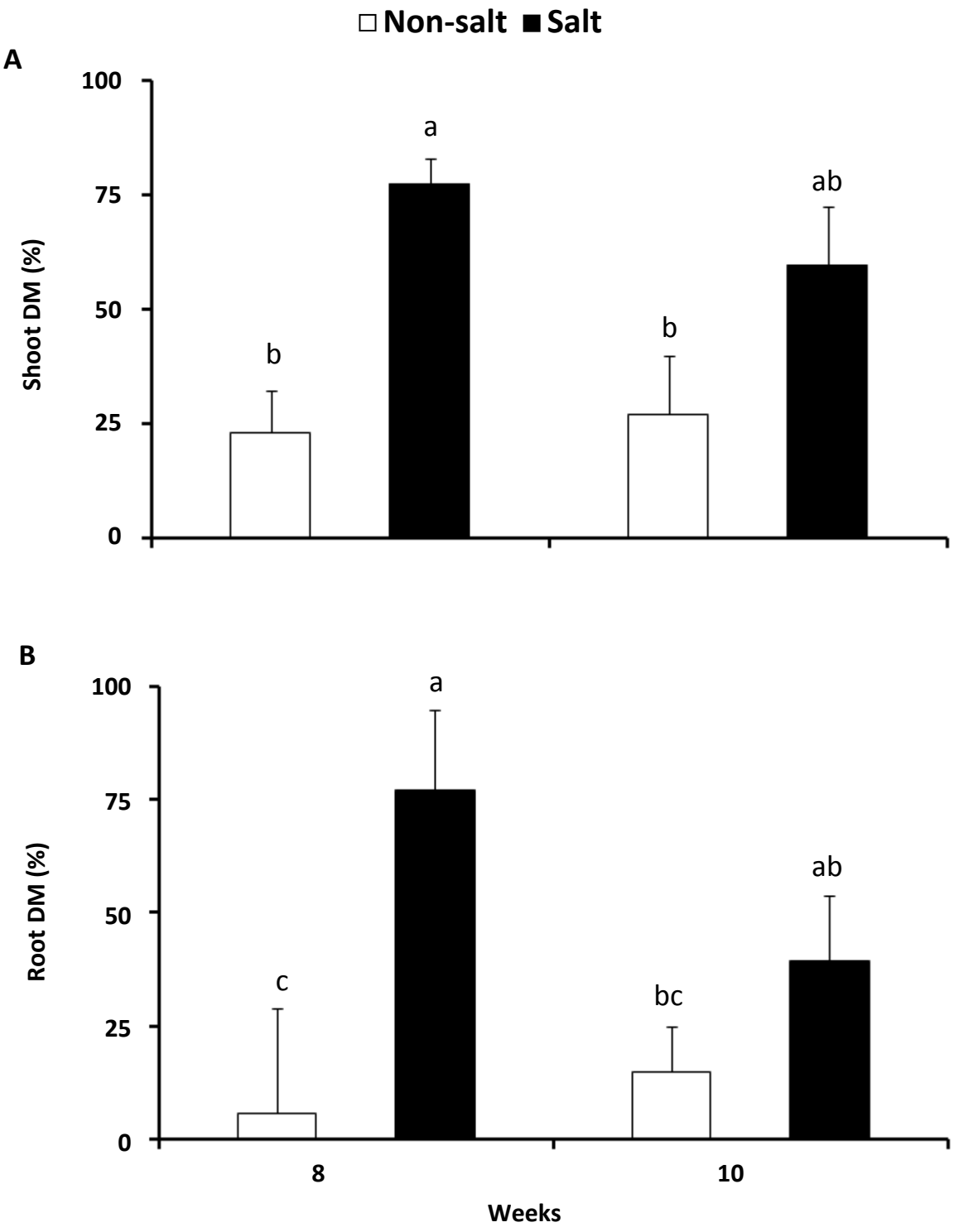

□ Non-salt ■ Salt
A
Shoot FM (%)
120
90
60
30
0
ab
a
b
a
B
Root FM (%)
250
200
150
100
50
0
b
a
a
a
8
10
Weeks

Figure 8: Effect (%) of AM over non-AM colonization on the dry mass (DM) of shoots (A) and roots (B) of tomato plants under non-salt or salt treatment at weeks 8 and 10. Means (n=5) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Tukey's test.


□ Non-salt ■ Salt
A
100
75
50
25
0
Shoot DM (%)
a
ab
b
b
B
100
75
50
25
0
Root DM (%)
a
ab
c
bc
8
10
Weeks

3.2 Second experiment

Mycorrhizal colonization and density

Salt (P=0.066) and time (P<0.001) had effects on AM root colonization in tomato. The colonization % was significantly higher in salt than non-salt treated roots after four weeks, i.e. 2.6% vs. 0% (Fig. 9). For the mycorrhizal density, the ANOVA showed a significant effect of time (P<0.001) and its interaction with salt (P=0.036). There was also a significant effect of time on hyphal, vesicular and arbuscular density (Figs 10-13). Overall, the mycorrhizal density increased with time regardless salt treatment.

Shoot and root fresh and dry mass and shoot height

The FM and DM of shoots increased overtime, and remained relatively similar regardless the AM or salt treatment within the same period (Figs 14 and 15). The ANOVA on shoot height detected significant effects (P<0.001) of both salt treatment and time. Regardless the AM and salt treatments, shoot height significantly (P<0.001) increased with time. There was a significant difference between salt and non-salt treated plants after eight weeks (Fig. 16). The shoot height under the salt treatment, regardless the AM treatment, was significantly smaller than the non-salt and AM treated plants at week eight and the non-AM non-salt treated plants at week ten.

The ANOVA on root FM showed significant effects of time (P<0.001) and AM (P=0.047) treatments and interaction of AM with salt (P=0.032). For root DM, significant

effect of time (P<0.001) and interactions of time with AM (P=0.048) and salt (P=0.015) were detected. However, the AM and salt treatments had no effect on root FM and DM within a similar period (Figs 17 and 18).

Chlorophyll concentration and mineral analysis

Overall, the concentrations of both chlorophylls *a* and *b* remained constant with time and there was no effect of AM or salt treatment (Fig. 19 A, B).

Sodium (Na)

In roots, significant (P<0.001) effects of salt, time and AM were revealed by ANOVA, and significant interactions of AM with salt (P=0.005) and time (P=0.022). Overall, Na concentration was significantly increased with time in AM plants regardless the salt treatment (Table 1). There was a threefold increase of Na concentrations in roots of salt than non-salt treated ones, and constant in non-AM roots over time. Na concentration in shoot was significantly influenced by salt (P<0.001) and time (P<0.001). In fact, the shoot Na concentration was higher in salt than non-salt treatment (Table 3). In non-salt treated plants, the shoot Na concentration decreased significantly over time.

K/Na ratio

The ANOVA on the K/Na ratio in shoot showed significant effects of salt (P<0.001) and time (P<0.001) and their interaction (P<0.001). In fact, the shoot K/Na ratio was significantly lower in salt than non-salt treatment and increased by four times

Figure 14: Shoot fresh mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment between week 4 and 10. Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Tukey's test.

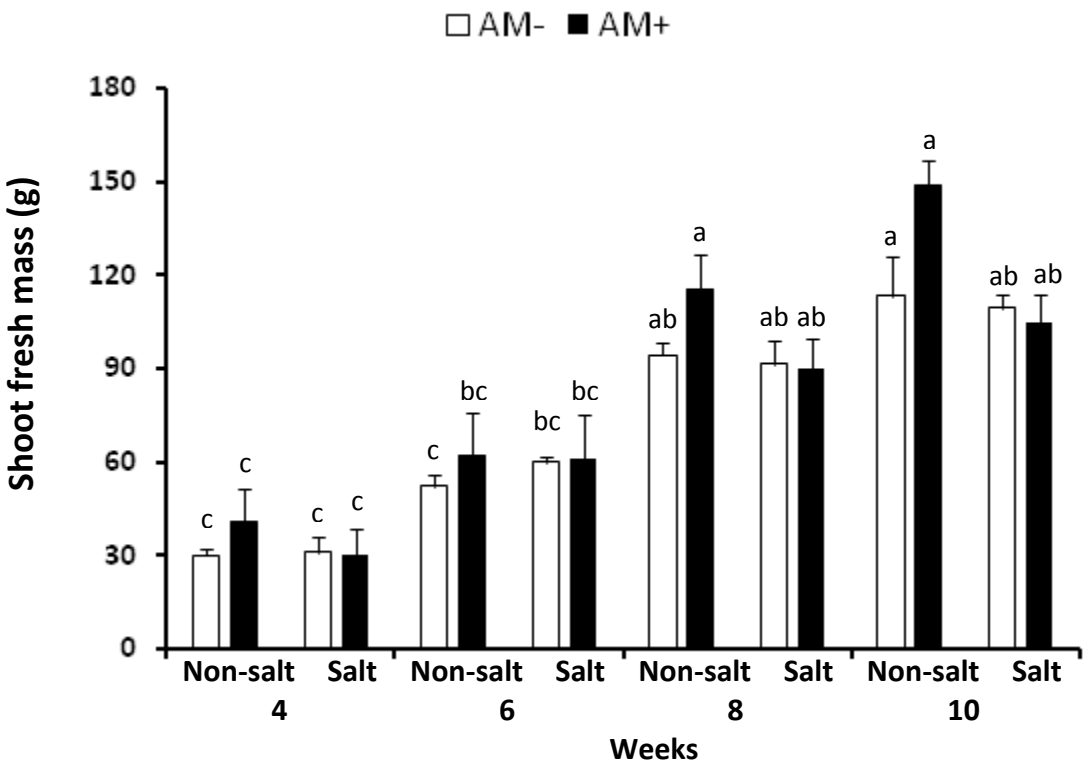

AM-
AM+
Shoot fresh mass (g)
180
150
120
90
60
30
0
c
c
c
c
c
bc
bc
bc
ab
a
ab
ab
a
a
ab
ab
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
4
6
8
10
Weeks

Figure 15: Shoot dry mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment between week 4 and 10. Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

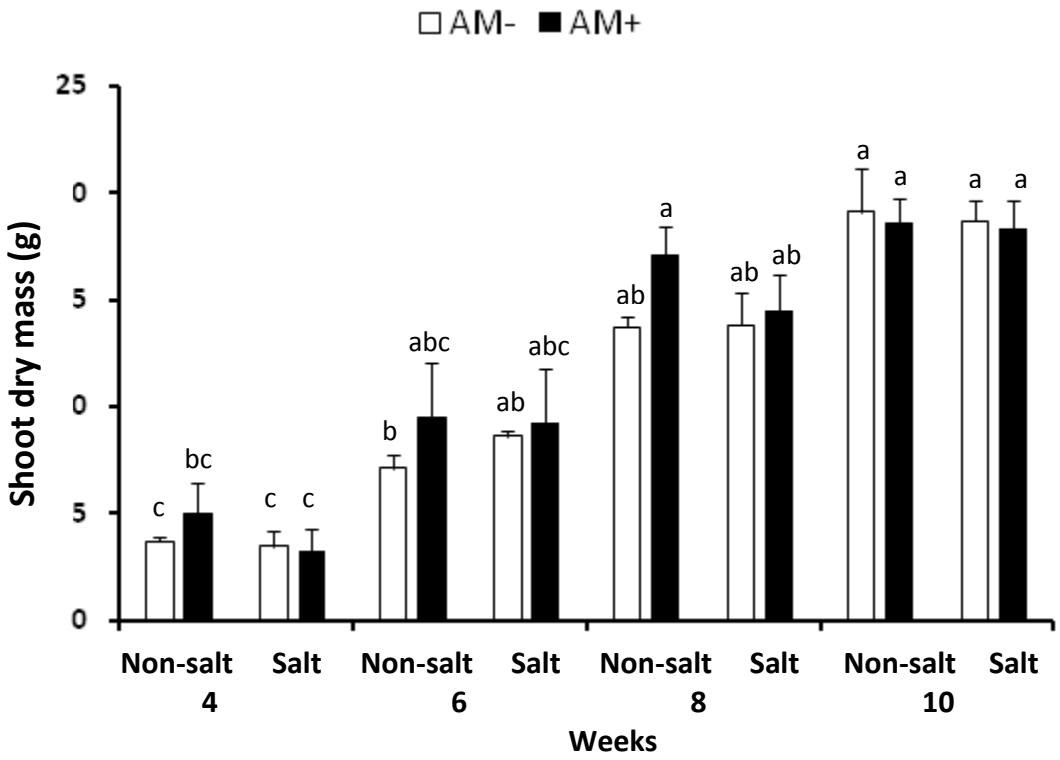

AM-
AM+
Shoot dry mass (g)
25
0
5
0
5
0
c
bc
c
c
b
abc
ab
abc
ab
a
ab
ab
a
a
a
a
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
4
6
8
10
Weeks

Figure 16: Shoot height of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment between weeks 4 and 10. Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

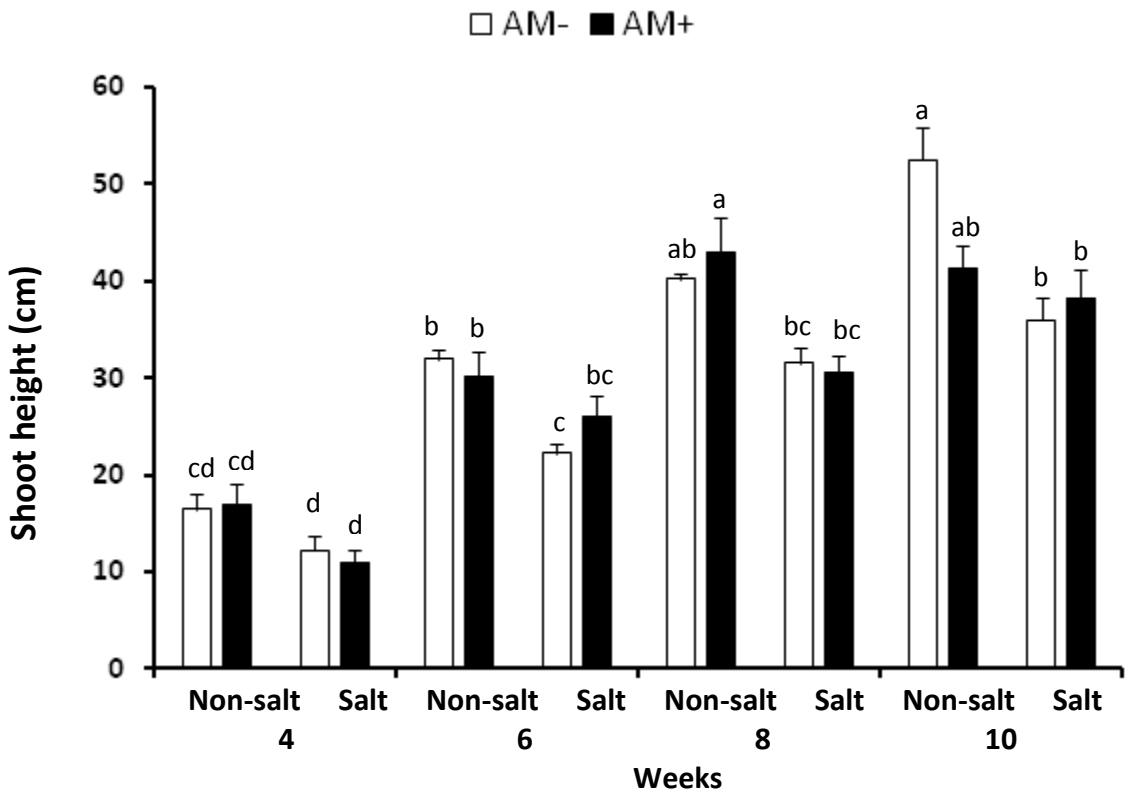

AM-
AM+
Shoot height (cm)
60
50
40
30
20
10
0
cd
cd
d
d
b
b
c
bc
ab
a
bc
bc
a
ab
b
b
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
4
6
8
10
Weeks

Figure 17: Root fresh mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment between weeks 4 and 10. Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

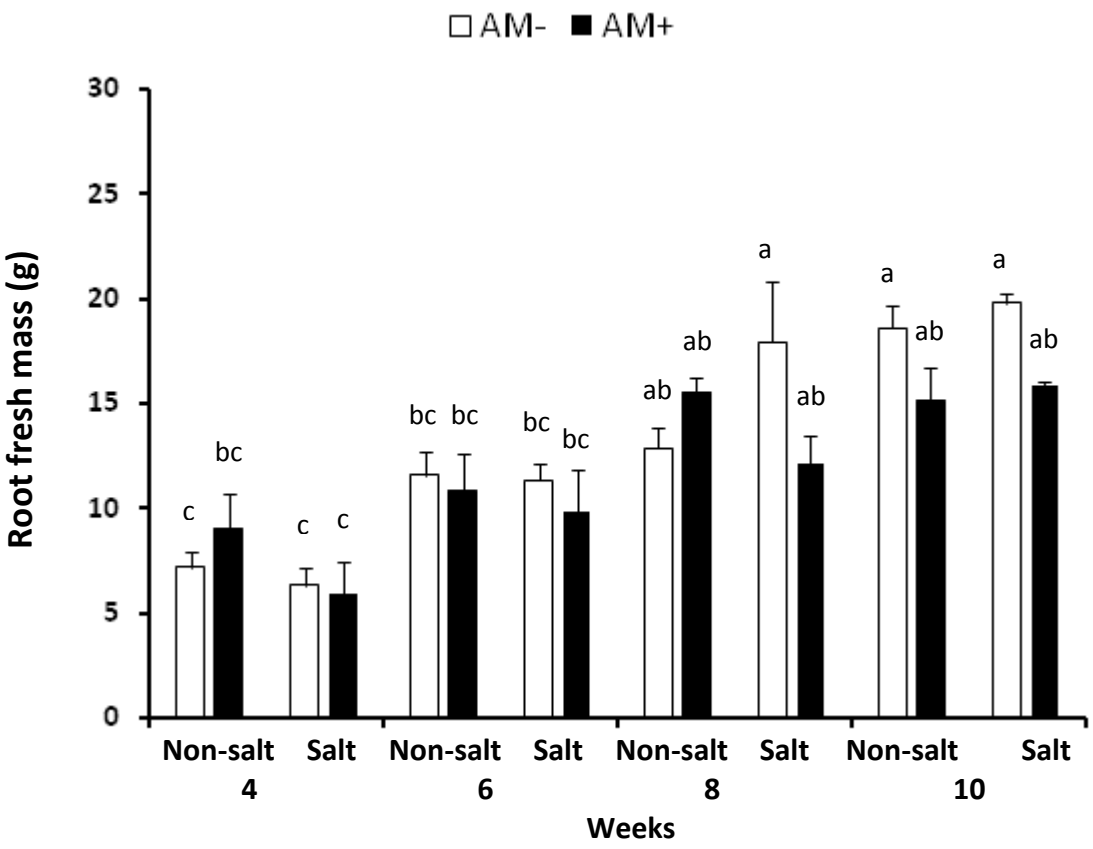

□ AM- ■ AM+
Root fresh mass (g)
30
25
20
15
10
5
0
c
bc
c
c
bc bc
bc
bc
ab
ab
a
ab
a
ab
a
ab
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
4
6
8
10
Weeks

Figure 18: Root dry mass of tomato plants with (AM+) or without (AM-) AM colonization under non-salt or salt treatment between weeks 4 and 10. Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

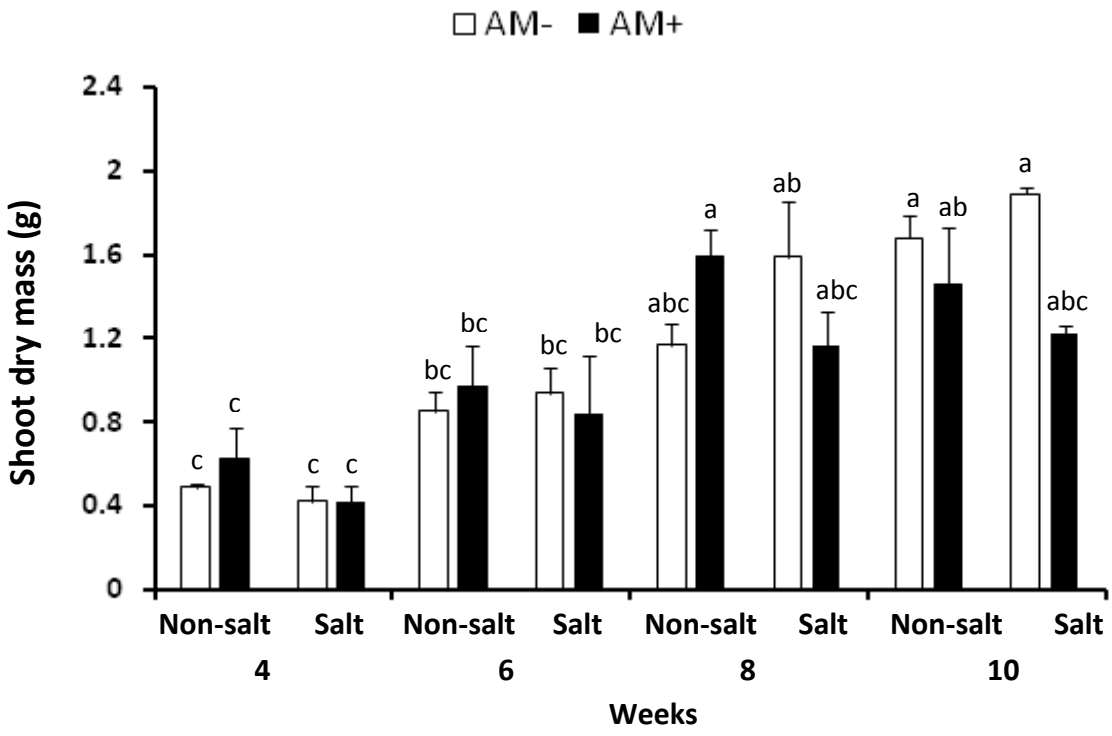
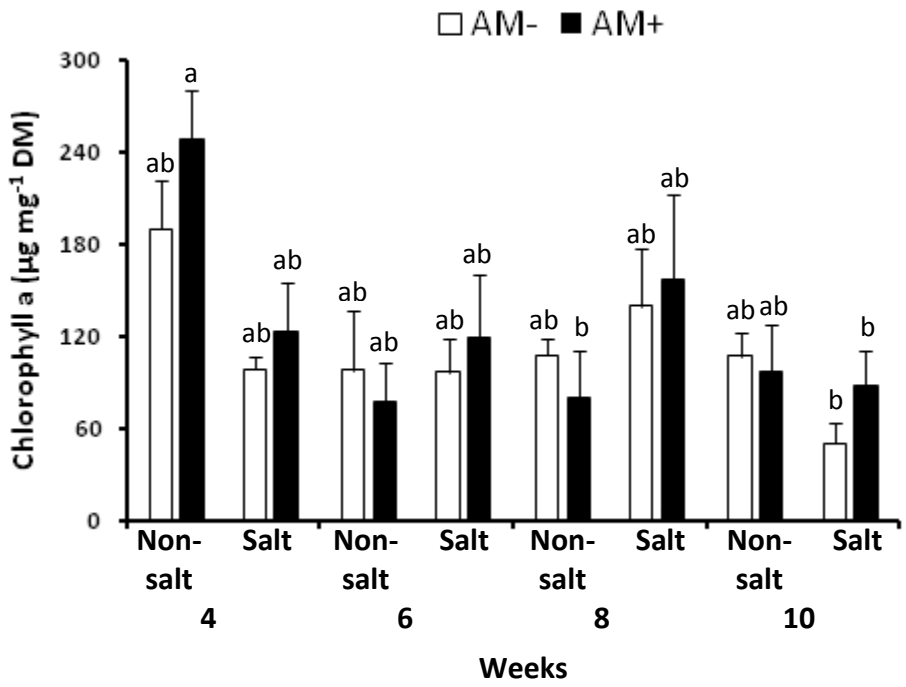

AM-
AM+
Shoot dry mass (g)
2.4
2
1.6
1.2
0.8
0.4
0
c
c
c
c
bc
bc
bc
bc
abc
a
ab
abc
a
ab
a
abc
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
Non-salt
Salt
4
6
8
10
Weeks

Figure 19: Chlorophyll concentration of tomato plants with (AM+) or without (AM-) AM colonization under non-salt and salt treatment between week 4 and 10. Means (n=4) and SE of chlorophyll a (A) and b (B) are shown. Bars sharing the same letters indicate treatments that are not significantly ($P<0.05$) different according to Bonferroni's test.

A.


AM-
AM+
Chlorophyll a (µg mg-1 DM)
300
240
180
120
60
0
Non-salt
Salt
4
6
8
10
Weeks

B.

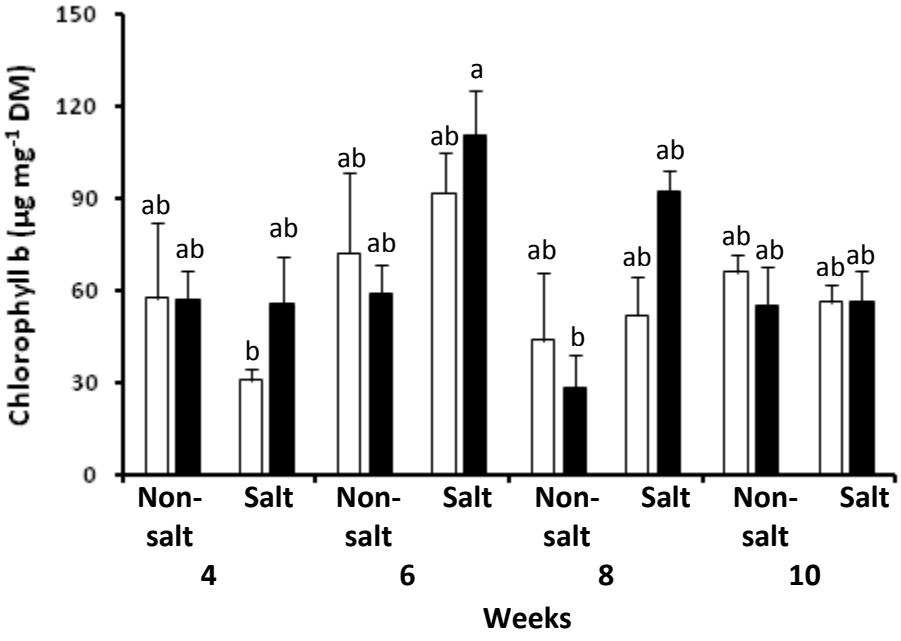
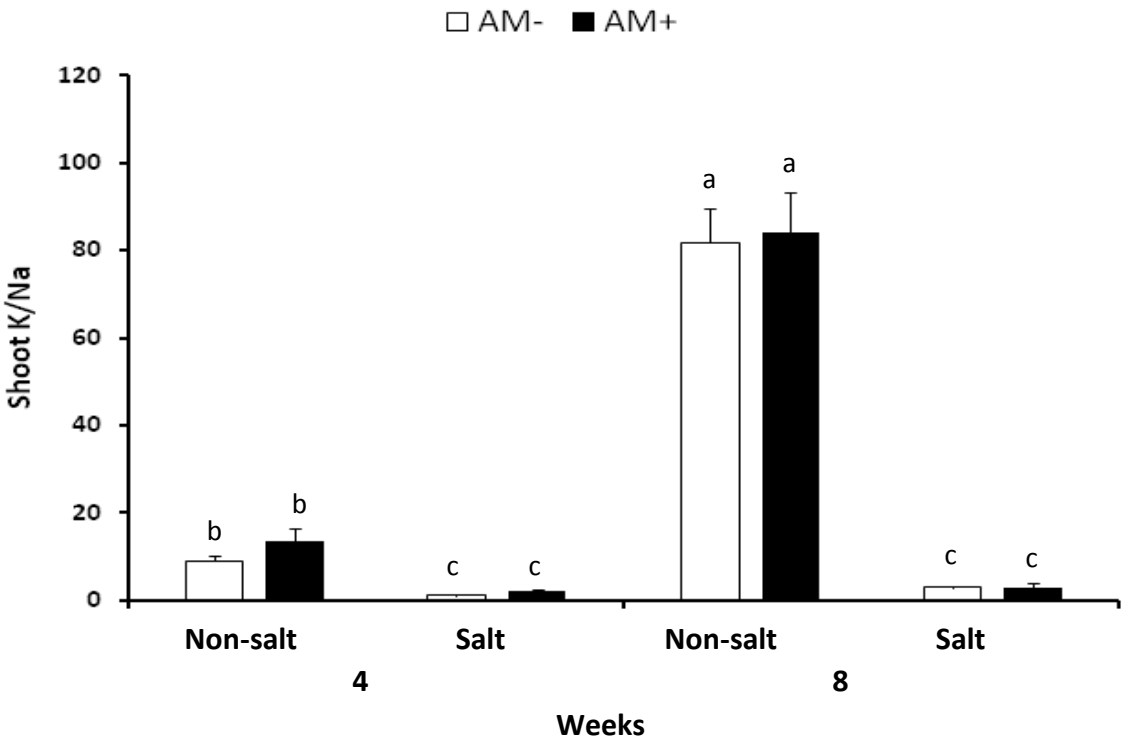
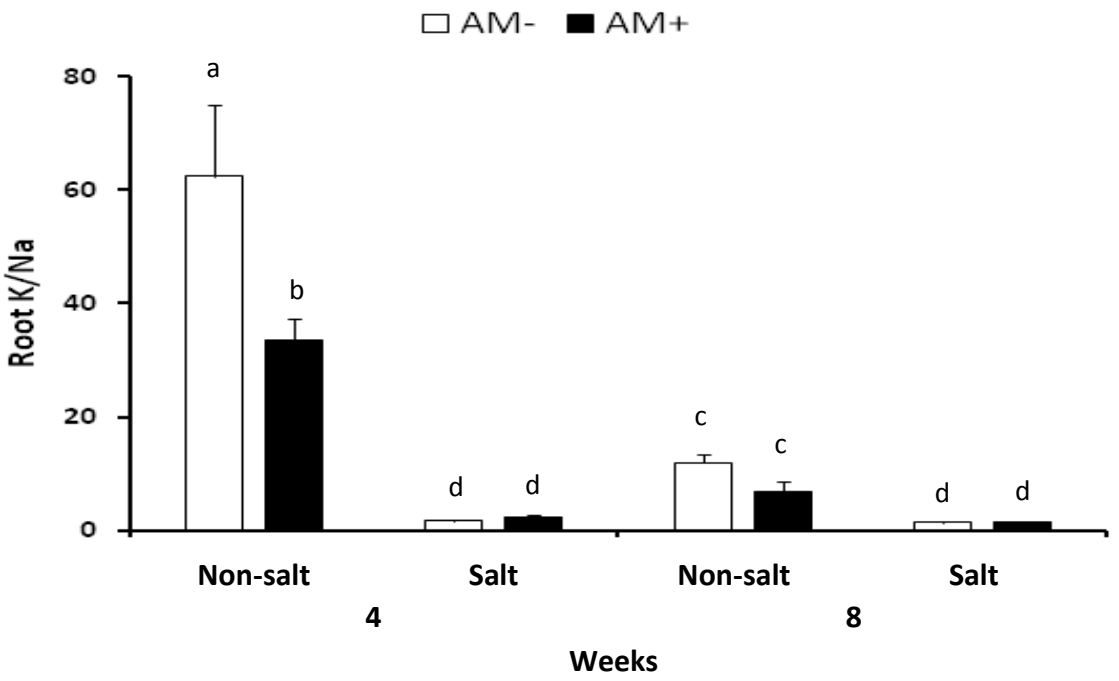

Chlorophyll b (µg mg-1 DM)
150
120
90
60
30
0
Non-salt
Salt
4
6
8
10
Weeks

Figure 20: Ratio of K/Na in shoot (A) and roots (B) of tomato plants with (AM+) or without (AM-) AM colonization under non-salt and salt treatment. Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Tukey's test.

A.


AM-
AM+
Shoot K/Na
120
100
80
60
40
20
0
a
a
b
b
c
c
c
c
Non-salt
Salt
Non-salt
Salt
4
8
Weeks

B.


AM-
AM+
Root K/Na
80
60
40
20
0
a
b
c
c
d
d
d
d
Non-salt
Salt
Non-salt
Salt
4
8
Weeks

in the non-salt treatment over time (Fig. 20A). In roots, the K/Na ratio was significantly influenced by AM ($P=0.047$), salt ($P<0.001$) and time ($P<0.001$), and significant interactions were detected between salt and AM ($P=0.0028$), and salt with time ($P<0.001$). The K/Na ratio in roots (Fig. 20B) decreased significantly over time in non-salt regardless the AM treatment, and was significantly lower in salt than non-salt treatments.

Calcium (Ca) and Potassium (K)

The Ca concentration in roots significantly decreased with time (Table 1), regardless the salt and AM treatments. By contrast, the K concentration was significantly lower in non-AM salt than non-salt treated plants. No difference was observed between AM-salt and non-salt treatments (Table 1). Only time had a significant effect ($P<0.001$) on shoot Ca concentration in shoot that significantly increased (Table 3) in all treatments. The K concentration did not change regardless of treatments (Table 3).

Phosphorus (P)

There was a significant interaction ($P=0.032$) of AM and salt treatments on P concentration in roots that was higher in AM than non-AM salt treated roots at week eight (Table 1). In shoot, the P concentration remained constant (Table 3).

Magnesium (Mg)

The effects of AM (P=0.001), salt (P=0.01) and time (P<0.001), and interactions of salt with time (P=0.005), and of the three variables (P=0.027) were significant for root Mg concentrations. At week eight, non-AM salt roots had significantly lower Mg concentration than all the other treatments and remained similar at week 4 (Table 1). In shoot, Mg concentration was similar among the treatments (Table 3).

Copper (Cu), Iron (Fe), Manganese (Mn) and Zinc (Zn)

For Cu in roots, the ANOVA showed a significant interaction between salt and time (P=0.02). In general, Cu concentration significantly increased over time in all treatments (Table 2). Only AM salt treated plants had a similar increase in Cu than non-salt. There were significant effects of AM (P=0.03) and time (P<0.001) on Fe in roots and interactions between salt and time (P=0.025). Overall, Fe concentration in roots significantly increased through time in all treatments (Table 2). Mn concentration in roots remained constant through time (Table.2). There were no significant effects of salt or AM treatments on Mn concentration. The ANOVA indicated a significant interaction between salt and time (P<0.001) for Zn concentration that significantly increased over time in non-salt treated roots and remained constant in the salt treated ones (Table 2). However, in non-salt treated plants there was a significant increase.

The ANOVA on Cu concentration in shoot showed significant effects of salt (P=0.006) and time (P<0.001). Overall, in both salt and non-salt treated shoots, Cu

concentration significantly decreased through time (Table 4). For Fe concentrations, there were significant effects of AM ($P<0.01$) and time ($P<0.001$), and significant interactions between AM and salt ($p<0.01$), and salt and time ($P<0.001$). Fe concentration in non-salt non-AM treated plants significantly decreased through time (Table 4). Time ($P<0.001$) and interactions between salt with AM ($P=0.012$) and salt with time ($P=0.039$) were significant on shoot Mn concentration that significantly increased in salt treated plants and remained constant in non-salt treated

Table 1: Mineral concentrations (mg g^{-1} DM) in root of tomato plants with (AM+) or without (AM-) colonization under salt (S+) and non-salt (S-) treatment after 4 and 8 weeks of growth.

Treatment	Weeks	Ca	K	Mg	Na	P
S-AM-	4	19.41 (1.58)a	19.10 (2.21)a	3.48 (0.12)c	0.33 (0.05)d	3.14 (0.38)ab
	8	7.53 (0.55)b	26.06 (4.12)a	5.68 (0.26)ab	2.35 (0.53)cd	3.40 (0.70)ab
S-AM+	4	19.26 (2.62)a	22.84 (1.91)a	4.32 (0.35)bc	0.70 (0.07)d	2.50 (0.72)ab
	8	8.69 (0.61)b	26.13 (5.04)a	5.98 (0.57)a	4.22 (0.85)c	3.18 (0.71)ab
S+AM-	4	21.54 (1.76)a	9.71 (1.66)b	3.80 (0.25)c	5.60 (0.44)bc	2.28 (0.34)ab
	8	5.95 (0.90)b	8.23 (1.01)b	3.40 (0.23)c	6.15 (0.76)bc	1.87 (0.19)b
S+AM+	4	19.49 (0.54)a	17.80 (2.94)ab	4.16 (0.25)bc	8.13 (1.04)b	2.32 (0.35)ab
	8	5.66 (0.34)b	17.06 (2.76)ab	5.48 (0.34)ab	12.17 (1.16)a	4.27 (0.42)a
F-values and p levels of significance Three-way ANOVA						
Mycorrhizae (AM)		0.0ns	6.0*	13.7**	27.6***	1.1ns
Salt (S)		2.1ns	24.1***	7.4*	141.8***	1.0ns
Time (T)		109.71***	0.9ns	24.7***	24.3***	2.9ns
AMxS		1.31ns	2.4ns	1.8ns	9.4**	5.2*
AMxT		0.24ns	0.1ns	0.2ns	5.9*	3.6ns
SxT		8.6***	2.1ns	9.3**	0.2ns	0.1ns
AMxSxT		0.02ns	0.2ns	5.5*	0.9ns	1.8ns

Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Bonferroni's and Tukey's test. ns: not significant

*p<0.05 **p<0.01 *** p<0.001

Table 2: Mineral concentrations (μg g^{-1} DM) in root of tomato plants with (AM+) or without (AM-) colonization under salt (S+) and non-salt (S-) treatment after 4 and 8 weeks of growth.

Treatment	Weeks	Cu	Fe	Mn	Zn
S-AM-	4	4.55 (0.12)c	57.40 (3.06)b	188.21 (12.81)a	22.91 (2.40)c
	8	44.75 (3.68)a	368.77 (146.90)a	141.75 (50.02)a	55.32 (4.29)ab
S-AM+	4	4.56 (0.37)c	82.01 (10.92)b	180.93 (73.67)a	22.97 (3.14)c
	8	51.83 (2.29)a	312.15 (53.21)a	225.67 (26.42)a	57.35 (7.19)a
S+AM-	4	4.01 (0.41)c	73.07 (2.43)b	249.90 (14.77)a	32.29 (2.28)bc
	8	31.36 (1.95)b	208.28 (10.50)a	164.75 (32.58)a	36.64 (2.09)b
S+AM+	4	5.09 (0.22)c	76.29 (1.76)b	193.16 (39.38)a	35.81 (2.95)bc
	8	45.38 (3.00)a	230.05 (33.48)a	215.74 (43.46)a	39.33 (2.86)b

F-values and levels of significance Three-way ANOVA

Mycorrhizae (AM)	15.5***	5.2*	0.3ns	0.3ns
Salt (S)	6.8*	0.1ns	0.5ns	0.0ns
Time (T)	2134.6***	117.7***	0.3ns	54.3***
AMxS	5.8*	1.6ns	0.5ns	0.3ns
AMxT	2.1ns	0.8ns	2.9ns	0.0ns
SxT	5.7*	5.6*	0.2ns	32.9***
AMxSxT	0.0ns	1.6ns	0.0ns	0.0ns

Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Bonferroni's and Tukey's test. ns: not significant

*p<0.05 ***p<0.001

Table 3: Mineral concentrations (mg g^{-1} DM) in shoot of tomato plants with (AM+) or without (AM-) colonization under salt (S+) and non-salt (S-) treatment after 4 and 8 weeks of growth.

Treatment	Weeks	Ca	K	Mg	Na	P
S-AM-	4	6.94 (1.08)b	25.63 (3.78)a	5.88 (0.25)a	2.96 (0.49)b	2.69 (0.33)a
	8	16.87 (2.60)a	20.89 (0.86)a	4.47 (0.27)a	0.26 (0.49)c	3.09 (0.13)a
S-AM+	4	5.89 (0.44)b	25.77 (5.58)a	6.17 (0.81)a	1.93 (0.21)bc	3.54 (0.79)a
	8	17.70 (1.39)a	23.77 (2.12)a	4.87 (0.42)a	0.29 (0.49)c	3.17 (0.32)a
S+AM-	4	5.56 (0.79)b	17.60 (4.18)a	5.05 (0.81)a	13.92 (2.08)a	3.42 (0.54)a
	8	20.52 (2.19)a	19.31 (1.37)a	4.95 (0.26)a	6.61 (0.49)ab	3.15 (0.21)a
S+AM+	4	5.01 (0.89)b	20.79 (6.83)a	4.03 (1.16)a	10.94 (3.18)a	2.50 (0.56)a
	8	16.73 (1.01)a	17.12 (3.11)a	4.55 (0.22)a	7.73 (0.49)ab	2.78 (0.41)a

F-values and levels of significance Three-way ANOVA

Mycorrhizae (AM)	1.1ns	0.12ns	0.2ns	0.9ns	0.0ns
Salt (S)	0.5ns	3.5ns	2.5ns	147.45***	0.2ns
Time (T)	161.9***	0.5ns	1.7ns	25.1***	0.0ns
AMxS	0.5ns	0.0ns	1.4ns	0.0ns	3.1ns
AMxT	0.1ns	0.0ns	1.1ns	3.0ns	0.0ns
SxT	2,6ns	0.1ns	3.1ns	1.8ns	0.0ns
AMxSxT	0.6ns	0.5ns	0.0ns	0.3ns	0.9ns

Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Bonferroni's and Tukey's test. ns: not significant

***$p<0.001$

Table 4: Mineral concentrations (µg g^{-1} DM) in shoot of tomato plants with (AM+) or without (AM-) colonization under salt (S+) and non-salt (S-) treatment after 4 and 8 weeks of growth.

Treatment	Weeks	Cu	Fe	Mn	Zn
S-AM-	4	31.65 (5.04)a	324.53 (63.32)a	140.14 (49.59)bc	51.13 (6.16)ab
	8	3.81 (0.33)bc	88.80 (5.73)b	218.31 (56.50)ab	20.92 (3.53)c
S-AM+	4	31.20 (2.78)a	277.85 (30.47)ab	117.60 (18.99)bc	43.79 (6.02)ab
	8	3.47 (0.43)bc	93.54 (5.84)b	295.28 (33.76)ab	23.63 (4.36)bc
S+AM-	4	21.34 (3.78)ab	229.17 (64.38)ab	108.22 (16.47)bc	41.50 (7.26)ab
	8	3.48 (0.22)bc	112.45 (3.67)b	394.87 (58.54)a	22.95 (1.34)bc
S+AM+	4	13.89 (3.79)ab	106.02 (19.69)b	46.00 (15.73)c	57.99 (6.78)a
	8	2.98 (0.29)c	89.52 (7.73)b	235.28 (36.43)ab	28.83 (9.27)bc
F-values and levels of significance Three-way ANOVA					
Mycorrhizae (AM)		3.98ns	7.6*	2.3ns	1.1ns
Salt (S)		8.75**	1.7ns	0.9ns	0.5ns
Time (T)		77.4***	68.3***	41.9***	32.9***
AMxS		1.6ns	8.9**	7.8*	2.5ns
AMxT		0.4ns	0.8ns	1.4ns	0.0ns
SxT		2.9ns	16.4***	4.7*	0.0ns
AMxSxT		0.3ns	0.0ns	0.0ns	1.4ns

Means (n=4) and SE are shown. Bars sharing the same letters indicate treatments that are not significantly ($P < 0.05$) different according to Bonferroni's and Tukey's test. ns: not significant

*p<0.05 **p<0.01 ***p<0.001

plants over time (Table 4). The ANOVA indicated a significant effect of time ($P<0.001$) on shoot Zn concentration that significantly decreased, except in salt treated plants.

3.3 Nested PCR detection

In the second experiment, I did a PCR analysis as a means to determine whether mycorrhization could be detected earlier than by the root staining method. Two sets of primers were designed to amplify specific fragments of the large ribosomal subunit of *Glomus intraradices*. The first primer pair, A1 and A2, amplified a DNA fragment of 822bp while the second pair, B1 and B2, amplified a fragment within the region of 423bp. After four weeks, PCR with either primer sets (A1-A2 and B1-B2) failed to yield any PCR product in non-salt and salt treated roots, however classical root staining showed AM colonization in one of the three salt treated plants. After six weeks, PCR also failed to amplify specific DNA from mycorrhization, even though root staining revealed it in all non-salt and salt treated roots (Appendices 2-5). PCR could successfully detect the presence of AM root colonization after eight weeks, where root colonization was at 63% and 40% in salt and non-salt treatments, respectively (Fig. 9). In non-salt treatment, the first set of primers (A1-A2) yielded an amplification product of the expected size 822bp in 2 of the 3 plants (Fig. 21A). However, root staining technique detected mycorrhization in all three plants. In salt treated plants, the first PCR produced bands in all three root samples (Fig. 21B). Interestingly, plant 1 which was highly colonized at 73% (Fig. 9) had the most intense bands. The nested PCR produced several bands including the expected size 423bp in both salt and non-salt treated plants. In addition, it was more sensitive in detecting mycorrhizal DNA than the first PCR and

amplified DNA in four out of nine of non-salt and in seven out of nine salt root segments (Fig. 22 A, B).

Figure 21: PCR amplification products from 8 weeks colonized tomato roots under non-salt (A) and salt (B) treatments. The primers A1 and A2 were used to amplify part of the large subunit ribosomal RNA of *G. intraradices*. Molecular weight of the ladder (L) is indicated as base pair. Positive (+) and negative (-) controls are shown.

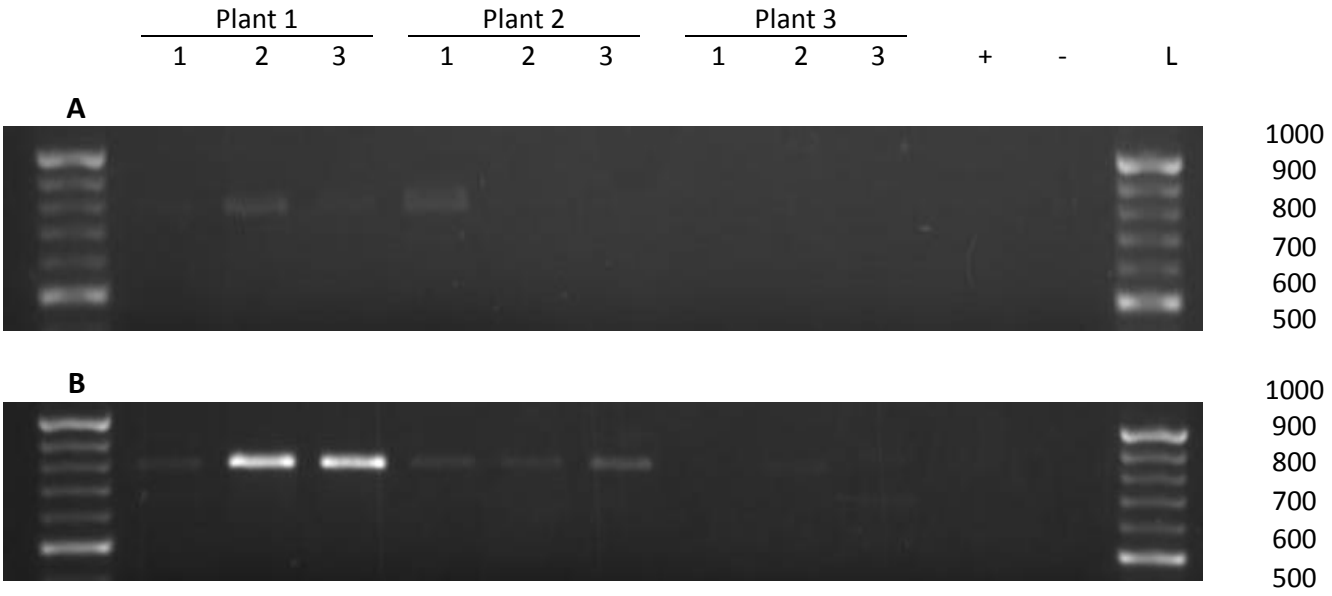
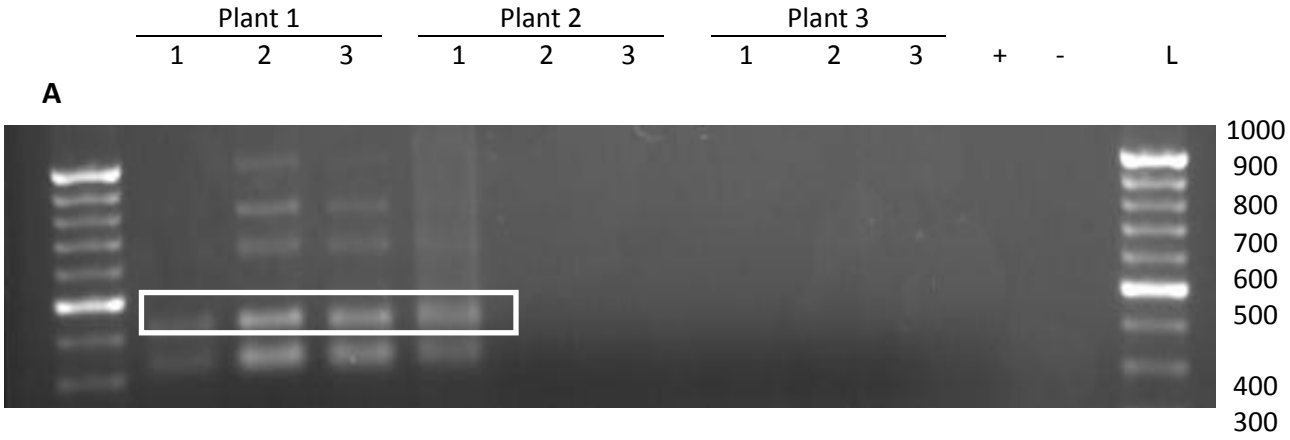

Plant 1
Plant 2
Plant 3
1 2 3 1 2 3 1 2 3 + - L
A
1000
900
800
700
600
500
B
1000
900
800
700
600
500

Figure 22: Nested PCR amplification products from 8 weeks colonized tomato roots under non-salt (A) and salt (B) treatments. The primers B1 and B2 were used to amplify part of the amplified PCR product from A1 and A2. Molecular weight of the ladder (L) is indicated as base pair. Positive (+) and negative (-) controls are shown.


Plant 1
Plant 2
Plant 3
1 2 3 1 2 3 1 2 3 + - L
A
1000
900
800
700
600
500
400
300


B
1000
900
800
700
600
500
400
300

Chapter 4

Discussion and Conclusion

In this research thesis, I have investigated the effect of colonization by the AM fungus (AMF) *Glomus intraradices* on the on growth and resistance of tomato plants under saline conditions. I have used an approach to subject simultaneously the plants and the AMF to salt stress. This allowed me to observe the early events of root colonization and responses of the plant and the AMF to salt stress. It was reported that root inoculation prior to salt exposure may stunt AMF and reduce root colonization levels (Hajiboland et al. 2010). This can be attributed to deleterious effects of salinity on AMF by reducing root colonization, delaying spore germination and limiting hyphal growth (Juniper and Abbott 2006).

Overall, increased root colonization level was observed in salt treated plants after eight weeks, however similar after ten weeks. This suggests that salt treatment accelerated the initial rate at which root colonization occurred. Furthermore, the level of colonization was related to the degree by which plants were alleviated from the deleterious salt effects. This implies that mycorrhization may increase plant capacity to adapt to the imposed salt stress, however not overcoming it.

Our findings support the first hypothesis stating that increased AM root colonization levels benefit plant performance under saline conditions. The effect of salinity on AM colonization was notable in this study and not always in agreement with general findings that show a decrease in root colonization with increasing salt stress. To

explain this difference, it is important to state that our saline conditions were maybe not high enough to hamper AM colonization, however high enough to stimulate plants to invest more in mycorrhization. Several authors showed in limiting-phosphate soils that plants invest in mycorrhization to overcome their nutrient needs (Bolan 1991; Cornwell et al. 2001; Jakobsen et al. 2001; Smith and Read 1997; Thingstrup et al. 2000). As phosphate levels in both salt and non-salt treatments were limiting may indicate that salinity might reduce mineral availability through precipitation in addition to direct stress in plants. It was shown that salinity reduces the availability of phosphates through ion-strength effects and [$Ca_3(PO_4)_2$] precipitation (Grattan and Grieve 1998). These effects may trigger early root colonization and AMF may help the plant to increase salt tolerance. To further explain this difference, it is noteworthy to distinguish between primary and secondary colonization (Wilson 1984). The primary colonization is dependent on spore germination, growth of hypha followed by the entry into the root (Bowen 1987); the secondary one is influenced by the host physiology and photosynthetate translocation (Smith and Read 1997). Given that salinity may cause several physiological stresses such as disruption of membrane, mineral imbalance (Evelin et al. 2009), lower photosynthesis and respiration rates, and inhibition of protein synthesis (Sheng et al. 2008; Zuccarini 2007), all of these can reduce carbohydrate allocation to support external hyphal growth. Consequently, it was presumed that AM colonization is higher in salt-treated plants due to internal growth where the host intercellular space is less exposed to salt where it can be sequestered in plant vacuoles.

Despite the limitation of secondary colonization, the primary colonization is a key factor in improving the plant salt tolerance.

The AM fungal efficiency can be measured by its contribution to plant growth under various environmental conditions (Ruiz-Lozano et al. 1996). In occurrence, the inoculation by *G. intraradices* helped tomato plants to maintain growth under the salt treatment imposed. To investigate the contribution of mycorrhization for alleviating salt stress, we examined the effect of salt stress on various physiological parameters. There is a variety of mechanisms by which AMF help plants to cope with salinity, *e.g.,* by improving nutrient uptake (Asghari et al. 2005; Cantrell and Linderman 2001), ion balance (Giri et al. 2007), enzyme activity such as nitrogenase in *Vicia faba* (Rabie and Almadini 2005), and water uptake (Ruiz-Lozano et al. 1996).

In the first experiment, an overall increase in shoot mass was observed when the salt treatment was applied after the appearance of the first true leaf. We also found an early AM effect in inducing growth of roots, later in shoot. Huang et al. (2010) found similar results with another tomato cultivar inoculated with *Glomus mosseae*. It was suggested by Schwab et al. (1982) that mycorrhization leads to greater carbohydrate allocation in shoot than roots. To ensure proper colonization, an initial resource allocation from the plant to the AMF usually occurs (Chapin et al. 1990; Crick and Grime 1987). Consequently, there is an increase in shoot/root ratio or a reduction in root mass (Smith et al. 2004). It was observed when comparing the response of two tomato

cultivars differing in salt tolerance, that AM colonization leads to more shoot than root mass as salt stress increases (Al-Karaki et al. 2001).

In the second experiment, the salt treatment was given the first week after seeds were germinated. As in the first experiment, we observed early root colonization, however its effect on growth was less pronounced likely because of the single salt application. This indicates that salt application procedure has an important impact on the overall outcome of root colonization. Our results suggest that salt stress accelerates and allows early root colonization.

As chlorophyll concentration was similar regardless the salt treatment, perhaps the salt level in our study was not high enough to detect any effect. It was reported that *Sesbania grandiflora* pigment content increases at low salt levels and tends to decrease at high levels (Dhanapackiam and Muhammad 2010). It was observed by Santos (2004) that sunflower plants exposed to high salt concentrations have a reduction in chlorophyll content and fluorescence, however increasing when exposed to lower salt level. Nutrient imbalance is commonly observed in salt treated plants (Grattan and Grieve 1998; Porras-Soriano et al. 2009; Zuccarini 2007). It was shown that tomato plants under salt stress have nutrient imbalance in Zn, P, Cu, K and Fe (Al-Karaki 2000) and that salt stress induces phosphorus deficiency in plants by reducing its uptake or translocation (Park et al. 2009). This loss in P uptake was attributed to Ca and H_2PO_4 precipitation in soil (Marschner and Dell 1994). Enhanced salt tolerance in plants was shown to be achieved by improving P nutrition (Colla et al. 2008). However, effects may

vary depending on the plant and type of salt used (Juniper and Abbott 1993). The AMF help in reducing yield loss due to salinity by increasing the uptake of low mobile ions (Al-Karaki 2000; Ruiz-Lozano et al. 1996), and improving plant water efficiency (Al-Karaki 1998). The enhancement of P uptake via mycorrhizal colonization is well known (Giri et al. 2007; Smith et al. 2004), and still considered among the main processes in improving plant salt resistance (Ruiz-Lozano and Azcón 2000). It was reported that supplementing P to tomato plants under salt stress increases their growth and decreases salt deleterious effects (Poss et al. 1985). However, it was also shown that salt tolerance of AM plants is independent of P concentration and more likely related to water content in zucchini (Colla et al. 2008), and to photosynthetic rate in maize (Feng et al. 2002). Another study with lettuce showed that alleviation of salt stress was mostly due to osmotic potential equilibrium capacity (Jahromi et al. 2008).

In our study, the concentration of phosphorous, regardless of any treatment, remained constant in shoot. By contrast, the P concentration in roots of salt treated AM plants was higher than in non-AM plants. This is in agreement with another study with tomato (Al-Karaki 2000). Increases in mineral uptake benefit plants in gaining biomass, then leading to a salt dilution growth effect. In addition, micronutrients such as Zn, Cu and Fe also contribute to overall plant health under saline conditions. Our results showed that AM plants under salt treatment kept their Zn level constant in roots, although it decreases in shoot. Overall, Cu concentration decreased in shoot when it increased in roots. Interestingly, Cu increased at a similar level in salt and non-salt treated AM plants. In salt treated non-AM roots, Cu concentration also increased but

not to the same extent as in non-salt treated plants. The AM colonization can enhance uptake of certain nutrients depending on saline conditions (Evelin et al. 2009). The Fe in salt treated plants remained constant through time. Surprisingly, Fe in non-salt non-AM plants decreased over time to the same extent as in shoot of salt treated plants. However, the level of Fe in roots increased similarly regardless of the treatment. These results are not in accordance with Al-Karaki (2000) who found greater Fe uptake in shoot of AM plants. In our study, salt was applied simultaneously to AMF inoculation presumably causing a delay in nutrient uptake via the external hyphae. It has been reported that external hyphal growth is delayed by salinity and in return slows nutrient uptake (Juniper and Abbott 2006).

We also looked at two important minerals which are involved in cell ion balance and salt stress signalling, *e.g.,* K and Ca. It has been shown that Na in plants has deleterious effects, such as disruption of ionic equilibrium, enzymatic disequilibrium, osmotic imbalance, cell division inhibition, and photosynthetic rate reduction (Mahajan and Tuteja 2005). In contrast to Na, where high levels are harmful to the plant, K is one of the ions that are needed in large amounts for plant homeostasis (Blatt 2000). It has been shown that K and Na compete for the same transporter binding site which decreases K uptake under saline conditions (Zhu 2003). It has been reported that by supplementing *Sorghum bicolor* with Ca, transport specificity towards K can be achieved (Colmer et al. 1996). Our results showed that Na levels remain constant in shoots and relatively high in salt treated plants and the highest in AM plants. Jahromi et al. (2008) observed that Na concentration in AM plants did not decrease presumably because

other salt tolerant mechanisms were initiated. In addition, AMF have not been associated with any Na exclusion mechanisms and many studies showed no decrease in salt uptake in their presence (Al-Karaki 2000; Evelin et al. 2009). Our results showed that K concentration was quite constant in shoot and varies slightly in roots. In fact, the AM salt treated roots had similar K level as in the non-salt ones; however, it was the lowest in non-AM treated roots. This K variation in roots which are directly exposed to Na was reported by Aziz and Khan (2001) who showed that Na ions compete with K ions binding site. Our results suggest that AM colonization has an effect in maintaining K level and to plant salt tolerance. Another effect toward salt tolerance is the accumulation of Ca that is known to play a role in stress signalling then contributing to salt adaptation (Quintero et al. 2002). In addition, Ca can increase K selectivity and consequently assure a higher K/Na ratio (Parida and Das 2005). Our results showed no mycorrhizal dependent difference in Ca concentration which increased with time in shoot while decreasing in roots. These results are in agreement with our K/Na ratios that remained similar in salt treated plants.

Despite the fact that AM colonization can increase the uptake of most minerals, under salt stress we saw some effects in AM plants mainly in P uptake and maintaining nutrient balance of some ions, *e.g.*, Mg, Ca and K. We also observed high Na and P uptake in roots suggesting that there might be a link between them. Increased uptake in P is the main building block for membranes may help plants sequester higher levels of Na as it is accumulated in vacuoles. An early AM symbiosis is critical for the plant when dealing with abiotic stresses. Our results indicated that the time lapse for AM root

colonization decreases when plants are subjected to salt. These results support our second hypothesis which states that the time course required for root colonization is shorter in salt than non-salt treated tomato plants. To further explore this, nested PCR was used to detect *G. intraradices*. PCR detected the presence of AMF DNA when root colonization was at its highest as assessed by standard staining techniques. However, PCR detection could not be achieved earlier than eight weeks. This difference is most probably due to the different colonization levels at these two periods, being much higher at week 8. Hence, this difference in sensitivity can be related to our sampling method. It was reported that many PCR inhibitors are present in plants (Pandey et al. 1996). Furthermore recent advances in DNA analysis suggest that AMF have high DNA variability which make PCR detection difficult in assessing species and more traditional means are required in their identification (Koch et al. 2004; Martin et al. 2008). Consequently, our results suggest that AMF root detection by PCR was not the ideal approach for early root colonization.

In conclusion, this study allowed us to determine combined effects of salt stress and mycorrhization on tomato plants. Interestingly, we observed an early root colonization under salt conditions. In the first experiment, this difference was more pronounced when salt was applied after the appearance of the first true leaf. Similar observations were made in the second experiment although with lessened effects. This difference may be due to the different inoculation procedures. Finally, my attempt to introduce molecular biology in order to determine early colonization was somewhat

successful, whereas not conclusive. Lastly, the early root colonization in salt treated plants suggests an early release of root exudates that stimulate colonization.

To expand from this research framework, field work study under similar conditions or the application of other AMF species could be prepared in order to explore other ways to increase salt tolerance in tomato plants.

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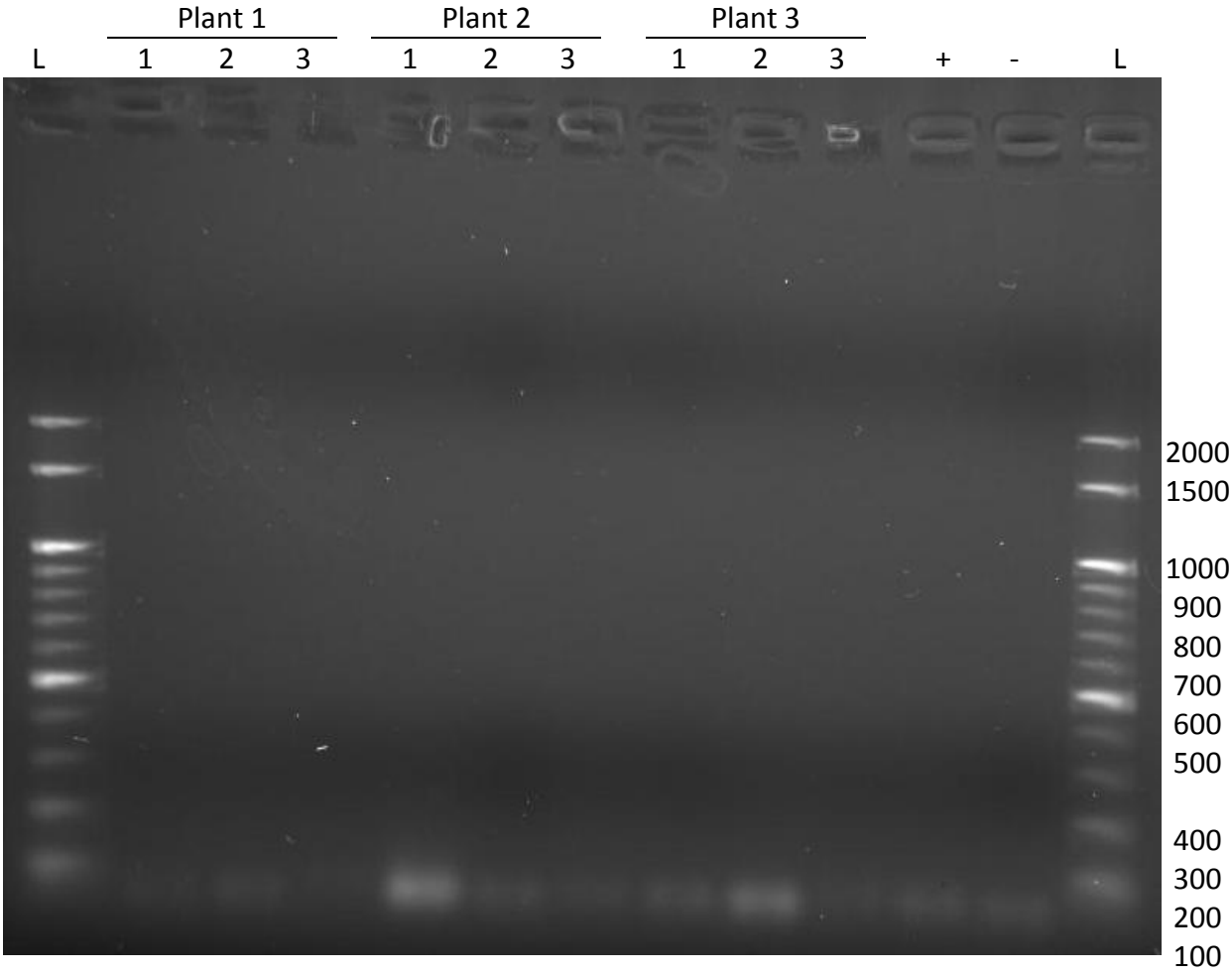
Appendices

Appendix 1 : Long Ashton nutrient solution.

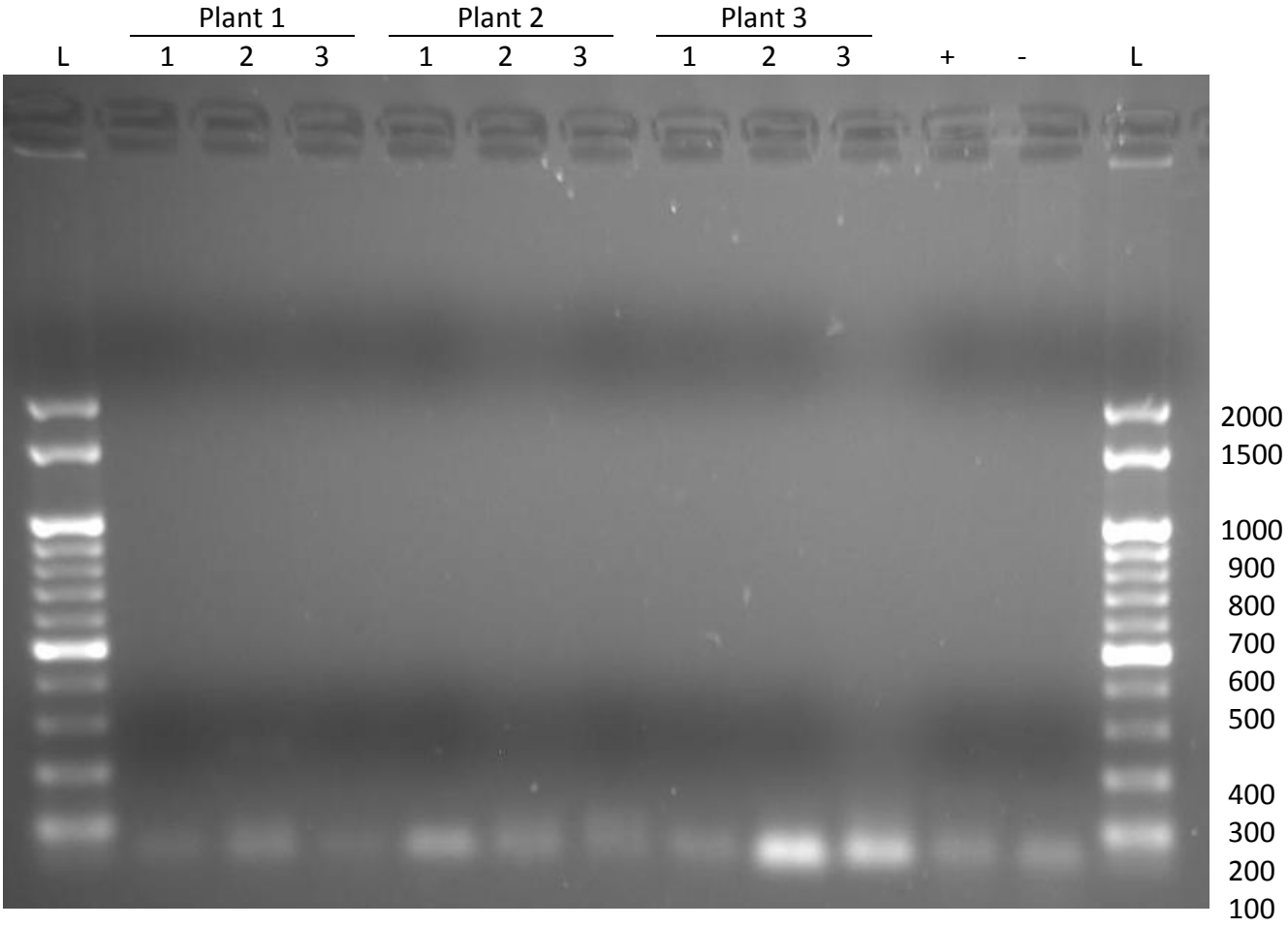
Macronutrient	MW	mM	g/L	Stock	g/L
K_2SO_4	174.26	2	0.35	500X	174.27
CaCl2	110.99	4	0.44	200X	88.1
MgSO4.7H2O	246.48	1.5	0.92	200X	184.86
NaH2PO4.H20	137.99	1.5	0.51	200X	103.49
NH4NO3	80.04	5µM	0.001	200X	0.2

Micronutrient	MW	mM	mg/L	Stock	g/L
MnSO4.H2O	169.02	0.01	4.2	200X	0.84
CuSO4.5H2O	249.68	1µM	0.6	200X	0.12
ZnSO4.7H2O	287.5	1µM	0.07	200X	0.014
H3BO3	61.83	0.05	7.7	200X	1.54
NaCl	58.44	0.09	13.15	200X	2.63
Na2MoO4.2H2O	241.95	5µM	0.3	200X	0.06
EDTA-Fe	367.1	0.1	91.75	200X	18.35

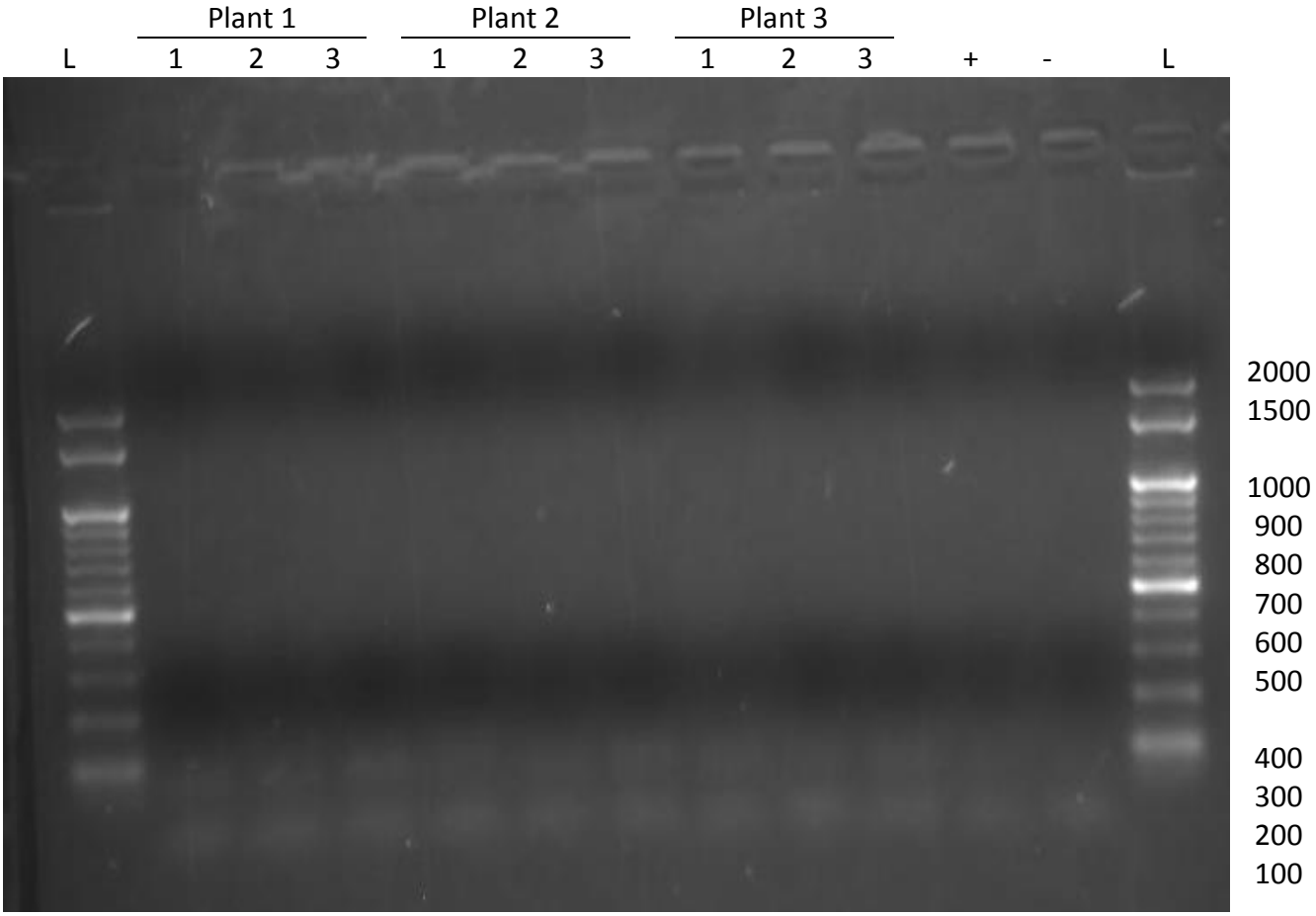
Appendix 2: PCR amplification products from 6 weeks colonized tomato root under non-salt treatment. The primers A1 and A2 were used to amplify part of the large subunit ribosomal RNA of *G. intraradices*. Molecular weight of the ladder (L) are indicated as base pair. Positive (+) and negative (-) controls are shown.


Plant 1
Plant 2
Plant 3
L
1
2
3
1
2
3
1
2
3
+
-
L
2000
1500
1000
900
800
700
600
500
400
300
200
100

Appendix 3: PCR amplification products from 6 weeks colonized tomato root under salt treatment. The primers A1 and A2 were used to amplify part of the large subunit ribosomal RNA of *G. intraradices*. Molecular weight of the ladder (L) are indicated as base pair. Positive (+) and negative (-) controls are shown.


Plant 1
Plant 2
Plant 3
L
1
2
3
1
2
3
1
2
3
+
-
L
2000
1500
1000
900
800
700
600
500
400
300
200
100

Appendix 4: Nested PCR amplification products from 6 weeks colonized tomato root under non-salt treatment. The primers B1 and B2 were used to amplify part of the amplified PCR product from A1 and A2. Molecular weight of the ladder (L) are indicated as base pair. Positive (+) and negative (-) controls are shown.


Plant 1
Plant 2
Plant 3
L
1
2
3
1
2
3
1
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2000
1500
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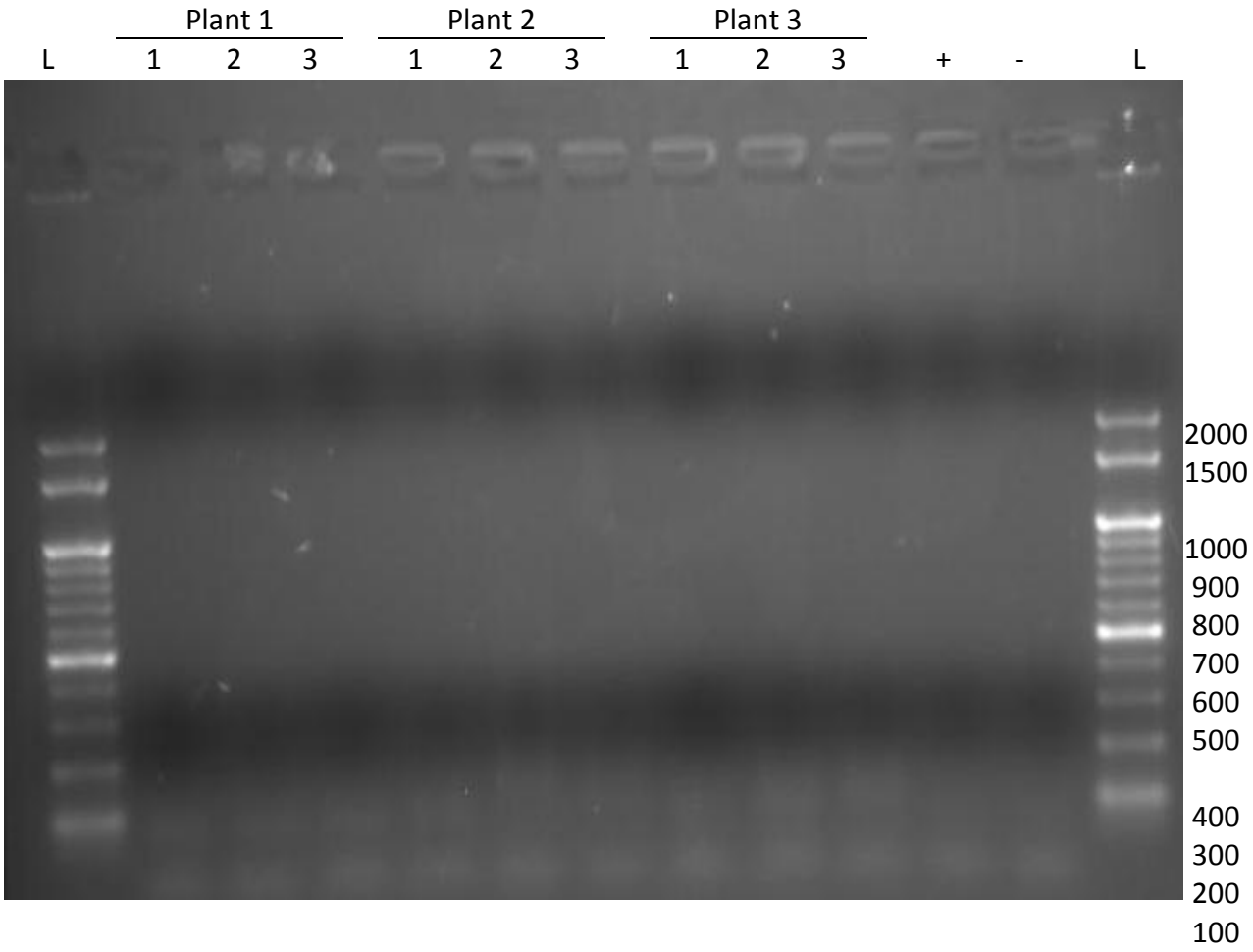
300
200
100

Appendix 5: Nested PCR amplification products from 6 weeks colonized tomato root under salt treatment. The primers B1 and B2 were used to amplify part of the amplified PCR product from A1 and A2 of plant. Molecular weight of the ladder (L) are indicated as base pair. Positive (+) and negative (-) controls are shown.


Plant 1
Plant 2
Plant 3
L
1
2
3
1
2
3
1
2
3
+
-
L
2000
1500
1000
900
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